STUDIES ON THE MICROBIOLOGY OF BARLEY MALT PRODUCTION

Hannah Itam Petters, M.Sc.

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DEDICATION

To my family

ABSTRACT

Populations of aerobic heterotrophic bacteria, mycelial fungi and yeasts occurring in the production of barley malt were examined by plating on agar media and by scanning electron microscopy. There was an increase in the total number of micro-organisms during germination of barley, although populations declined after kilning. Bacteria dominated numerically in all samples, with progressively lower populations of yeasts and filamentous fungi. There was no obvious pattern of spatial distribution of micro-organisms on/in the samples, although there appeared to be high populations of bacteria and fungal hyphae on the inner surface of kernels. The dominant groups of aerobic heterotrophic bacteria were presumptively identified as Alcaligenes sp., Arthrobacter globiformis, Clavibacter iranicum, Erwinia herbicola, Lactobacillus spp. and Pseudomonas fluorescens. The principal filamentous fungi were Alternaria alternata, Aspergillus glaucus group, Cladosporium macrocarpum, Epicoccum purpurascens, Fusarium avenaceum, Geotrichum candidum and Penicillium spp. The yeasts isolated most frequently were Candida catenulata, C. vini, Debaryomyces hansenii, Hansenula polymorpha, Kloeckera apiculata, Rhodotorula mucilaginosa, Sporobolomyces roseus and Trichosporon beigelii. Representative bacteria, mycelial fungi and yeasts were examined for the ability to degrade β-glucan, starch or arabinoxylan. Approximately 50% of the fungi, <50% of the bacteria and <25% of the yeasts degraded these substrates. A culture filtrate of F. nivale demonstrated marked ability to reduce β-glucan viscometrically and colorimetrically. The organism also degraded raffinose and sucrose. In micro-malting experiments the addition of Fusarium nivale and

Geotrichum candidum did not produce substantial changes in terms of the physical and chemical characteristics of the finished malts.

INTRODUCTION

The Malting Process

As defined by Briggs et al. (1981), malt is a cereal grain, usually barley, which has germinated for a limited period, and then dried. It represents a rich source of carbohydrate, degraded protein, various vitamins and inorganic material. In addition, there is an abundance of enzymes, particularly amylases, unless the kilning process has been severe such as in the making of highly coloured malts.

The malting process may be divided into five main stages: the collection and storage (until required) of suitable barley, steeping of the grain in water, and thence its germination, drying and curing the resultant malt in the kiln, and finally screening of the dried product.

Barley is grown under widely varying conditions. Consequently, the quality of grain varies considerably. Hence, maltsters are wary in selecting barley to meet their specific requirements, including a moisture content of 9 - 25%. The grain should be of even appearance, containing only a few damaged, cracked or half-kernels. There should be no pre-germination and it should not be stained or weathered (discoloration is often regarded as the sign of fungal attack). The grain must smell and taste 'clean', having no trace of bitterness, sweetness or mustiness, or other character that indicates heavy microbial contamination. A sample should contain not more than small quantities of contaminants, such as weed seeds, other cereal grains, stones, nails, straw and other detritus. Unfortunately, not all forms of grain deterioration may be detected by visual inspection. After such inspections, the grain is stored at ambient conditions until required for malting.

The next stage involves steeping the grain in water so as to raise the moisture level from 11 - 12% to 42 - 48% (fresh weight basis). This level is sufficient to support 'modification', but will not allow excessive growth. 'Modification' is a term used to describe the biochemical and physical changes in the grain occurring during germination. The changes are essentially: (1) A large increase in the quantities of some of the hydrolytic enzymes present in the grain; (2) A partial degradation (hydrolysis, catalysed by enzymes) of reserve substances, i.e. cell walls, gums, protein and starch, in the starchy endosperm. Consequently, there is a loss in dry weight of the malt compared to the dry barley.

In some cases, steeping is not continuous, but interspersed with air rests to speed up the germination of the grain. With this process, air is drawn through the grain bed. The period of wetting and air resting varies with different maltings.

The third phase of malting is germination. Here moist barley is allowed to grow under controlled cool conditions (12 - 18° C) in a humid atmosphere, normally without any further addition of water. The grain is turned daily during germination to create an even temperature in the bulk, to prevent matting of rootlets, and to promote good air ventilation. The duration of germination (usually 5 - 7 days) varies between maltings, reflecting the state of barley corns and the desired characteristics of the final product. For instance, brewer's malt requires a shorter germination period than a product destined for distilling. Additives, such as growth hormones, may be added to stimulate rootlet growth.

At the end of germination when the barley is fully modified, the green malt is dried in a kiln equipped with a hot air flow. Here, the

aim is to achieve a moisture content of 1.5 - 6.0% fresh weight. Kilning not only dries the malt, but it also inactivates enzymes, and develops colour and flavour as well as removing volatile compounds. Peat is often burned in the kiln to impart flavour to distillers' malts. Kilning is usually done in an incremental temperature range (50 - 75° C).

The kilned malt is passed through screens to remove culms (coleoptiles) and rootlets, which are subsequently used for cattle food, and the screened malt sent to store (Briggs et al., 1981).

The traditional floor malting and the modern pneumatic saladin system are similar in their principles of operation and in the nature of the resultant products. However, in traditional floor malting, steeped barley is 'cast' onto an impervious floor to allow germination as a 10 -15 cm thick layer, before transferance to a separate vessel for kilning. In contrast, compartmentalised boxes, named saladin boxes, are used in pneumatic maltings (Briggs et al., 1981). The depth of the saladin box may be up to 2 m, and the malting barley is cast onto its perforated floor. The enormous size of the box invariably calls for helical screw turners, which turn the barley during malting.

Whether the finished malt is from floor maltings or saladin boxes, it may be destined to serve as one of the raw materials for the production of beer, whisky, wine vinegar or other related beverages and food stuffs.

Usually, a brewer uses, as his primary raw materials, malted barley, water and hops. The malted barley is milled and mashed to obtain an aqueous extract. There are several methods of mashing to obtain a satisfactory wort, one of which is the simple infusion mashing system, common in Britain (Briggs et al., 1981). Infusion mashing

entails the intimate mixing of the ground malt with hot water. The quantity of temperature controlled water is regulated in such a way as to produce a final temperature in the mash of approximately 65° C. This infusate has a thick porridge-like consistency. The temperature is maintained for periods of 30 min. to several hours, a period during which starch is enzymatically converted to fermentable sugars, and limit dextrins (amylolysis). The sweet wort is then extracted in a mash tun (a vessel which has a slotted base), which acts like a sieve. In order to wash all the sweet wort from the spent grains, an additional wash with hot water (this is referred to as hot liquor by brewers) at 79° C is carried out. The clarified wort is boiled with hops in a copper or kettle in order to stop enzymic action, and to sterilize the wort, remove some of the proteinaceous materials as a coarse coagulum (hot trub), and speed up the desirable chemical changes. The optimum duration of boiling from the point of view of bitterness is ~90 min. The hopped wort is centrifuged to remove the hot trub and other insoluble materials. Thence, this clear hopped wort is cooled, and further protein and tannin materials are precipitated as 'fine break' (cold trub).

The cooled, aerated, clear, hopped wort is now fermented with pressed yeast (1 - 2 g l^{-1} wort) at ~16° C for top yeast and 10° C for bottom yeasts. The yeasts grow using amino acids of the wort as the main source of nitrogen, and the glucose, fructose, sucrose, maltose and maltotriose as the principal source of carbon and energy. The yeasts multiply to 5 - 10 times the original weight, and demonstrate an overwhelmingly fermentative metabolism. Thus, the end products are mainly ethanol and CO_2. Fermentation is complete within 2 - 10 days, largely depending on temperature.

The fermented wort is separated from the yeast by centrifugation or filtration. After partially flushing out the volatiles with CO_2, fermentable sugar is added and the temperature gradually reduced to 2° C for maturation. Finally, the beer is filtered and dispensed into bottles, or kegs/casks. Pasteurization is usually carried out to ensure sterility.

Brewers are interested in using a well modified malt that will pose little or no problem in brewing. Although the introduction of modern technology has shortened the period required to produce a well-modified malt, there is sufficient time and nutrients available for the natural microbial contaminants of barley to grow on the fully hydrated grains, as during malting. Yet very little work on the microbiology of commercial malting has been published.

Micro-organisms Associated with Barley, Malt and Beer

The nature and the magnitude of the microflora of barley depend on both the field conditions under which the crop was grown and the post-harvest history of the grain.

As in wheat (Flannigan & Campbell, 1977), barley kernels are first contaminated by air-borne bacteria, yeasts and moulds soon after the ears emerge from the developing leaf-sheaths (Hill & Lacey, 1983a). Warnock (1973) found that the number of Cladosporium spores deposited on the outside of the lemma and palea is related to the populations in the atmosphere. Moreover, levels increase markedly after rain. When the inside surface of the bracteoles is exposed, the Cladosporium spores germinate and spread to form a superficial mycelium, which later invades the parenchyma layer of the lemma and palea (Warnock, 1973).

Mycelia of other field fungi such as Alternaria (Warnock, 1973) and Cochliobolus (= Helminthosporium; Mead, 1943) have also been noted on the inner surface and within the bracteoles. The pericarp layer of the caryopsis may be extensively invaded by mycelia (Mead, 1943; Warnock & Preece, 1971), but, perhaps less so than the lemma and palea (Tuite & Christensen, 1955). Cladosporium mycelium, which invades bracteoles and the upper layer of the caryopsis, may originate in trapped anthers (Warnock, 1973). Alternaria alternata, C. cladosporioides and C. herbarum have been reported to penetrate undamaged living host tissues mainly via stomatal apertures (O'Donnell, 1980). A. alternata and C. herbarum have been reported as pathogens of barley and maize, respectively (Dickinson, 1981). C. cladosporioides has been implicated with wheat head blackening (Bancroft, 1910). The components of the microflora of ripening grain have been listed frequently (see Pepper & Kiesling, 1963; Mislivec & Tuite, 1970; Flannigan, 1974). It would appear that the most abundant filamentous fungi are A. alternata, Epicoccum purpurascens and Verticillium lecanii (Hill & Lacey, 1983a).

Under humid weather conditions in spring, Pseudocercosporella herpotrichoides, Rhizoctonia cerealis and different Fusarium spp., i.e. mostly F. culmorum, F. avenaceum, F. graminearum and F. nivale, are reported to be involved in the foot-rot complex of wheat, barley and rye (Reinecke, 1981). Fusarium spp. are often seed-borne on cereals and, therefore, colonize ears and grains. This colonization may be inhibited by Alternaria sp. (Bateman, 1979). The saprophytic colonization of wheat and barley by Pyrenophora tritici-repentis was examined in the field. Here, the wheat and barley cultivars were exposed to a natural inoculum. As a result, all cultivars permitted recovery of the fungus (Summerell & Burgess, 1988).

Bacteria multiply rapidly in the vicinity of films of water on leaves (Leban & Draft, 1976). By dilution plating, it was revealed that during the early stages of development of barley kernels in the field, the microflora is numerically dominated by bacteria (Kotheimer & Christensen, 1961; Follstad & Christensen, 1962; Hill & Lacey, 1983a). Indeed, as many as 10^8 bacteria g^{-1} of barley have been isolated by Kotheimer & Christensen (1961) at the late dough stage. However, no detailed account of the bacterial flora has been published, although Erwinia herbicola and Xanthomonas campestris are often mentioned as being prevalent in pre-harvest barley (Clarke & Hill, 1981; Flannigan et al., 1982). Generally, the counts of actinomycetes in field grown barley are low (Clarke & Hill, 1981; Hill & Lacey, 1983a). Here, the dominant group appears to be Streptomyces spp. (Hill & Lacey, 1983a), and in particular S. griseus, S. albus and S. thermoviolaceus. Thermoactinomyces vulgaris, one of the causative agents of farmer's lung, may also be evident in the ripening and harvested barley (Flannigan, 1970; Clarke & Hill, 1981; Hill & Lacey, 1983a).

Detailed studies on the microflora of yeasts have not been published. However, a few studies have indicated that by harvest time barley is contaminated frequently by Sporobolomyces, and Rhodotorula (Lund, 1956; Clarke et al., 1966; Flannigan, 1974; Clarke & Hill, 1981). In addition, Hansenula, Torulopsis and Candida have been isolated from Danish barley before harvest (Lund, 1956). Cryptococcus spp. were also considered to be prevalent by Clarke et al. (1966). Although Kotheimer & Christensen (1961) reported the presence of Saccharomyces in pre-harvest barley in Minnesota, it is generally regarded that members of this genus are rare in barley (Pepper & Kiesling, 1963). Considering the close resemblances between the

filamentous fungal flora of barley and wheat (Flannigan, 1970a) it is likely that the species of Cryptococcus, Torulopsis and Trichosporon noted in pre-harvest wheat (Flannigan & Campbell, 1977) may also be present in barley (Flannigan, 1987).

Microbiological Contamination During Storage

Barley grain may be stored for a long period without deterioration providing the water content is <13.6% (Snow et al., 1944). Since many farmers do not have the capacity to satisfactorily dry barley for malting, grain merchants and maltsters often prefer to dry the grain themselves. Hence, by having specifications which state that, for example, 'Grain must be sound, sweet and free from heat, (visible) moulds and infestation' (Dolan, 1979), the maltster seems to safeguard his interests.

In storage of naturally-contaminated oats, wheat and barley seed over a 10 year period, Machacek & Wallace (1952) found that while the germinating ability was retained, viable field fungi effectively disappeared. Moreover, Fusarium spp. declined comparatively faster than A. alternata and Cochliobolus sativus.

The development of so-called storage fungi appears to coincide with the decline of field species. Thus, Mills & Wallace (1979) noted that the numbers of kernels bearing the predominant field fungi A. alternata and Cladosporium spp. apparently declined as those contaminated by Aspergillus glaucus group and Penicillium verrucosum var. cyclopium increased. These data were collected for piles of undried grain after a wet harvest in Manitoba. Micro-organisms associated with moist barley storage in unsealed silos included:

Endomycopsis chodatii, Hansenula anomala, Penicillium spp., Absidia spp. and Mucor pusillus, Asp. fumigatus, Humicola lanuginosa (Lacey, 1971). The numbers of yeasts may increase from 5 x 10^3 to 2.2 x 10^4 per kernel (Murphy, 1980).

Information on bacteria in stored barley grain is again limited. The list of bacteria on barley kernels given by Pepper & Kiesling (1963) includes Brevibacterium linens, Corynebacterium sp., Erwinia rhizogenes and Bacillus polymyxa. However, there was no indication whether these bacteria were from stored or field barley.

Micro-organisms of Malting Barley

During steeping of grain, supposedly dormant micro-organisms are activated, mould spores germinate, mycelia grow and yeasts and bacteria multiply. In commercial malting, Douglas (1984) found that where steeping is interrupted by an air-rest there may be increases of 4- to 25-fold in bacterial numbers in the grain by the end of the first steep. More dramatic increases of 35- to 150-fold occurred by the time the grain was couched. The corresponding increases in yeast numbers may be 3 to 20 and 5- to 1,300-fold. Sheneman & Hollenbeck (1960) noted that whereas total bacterial numbers increased from approximately 10^6 g^{-1} to 7 x 10^6 g^{-1} during steeping, mesophilic lactic acid bacteria showed greater increases of from 0 - 10^2 g^{-1} to >5 x 10^5 g^{-1}. Douglas & Flannigan (1988) also reported that mesophilic bacteria predominated at all stages of the malting process, and that viable counts of bacteria on green malt were 85- to 600-fold greater than on the original barley, but fell to less than the original level with kilning. They noted that Geotrichum candidum, a yeast-like species

rarely found in dry barley (Pepper & Kiesling, 1963) was prominent in green malt whereas Mucor spp. proliferated during kilning.

Laboratory malting experiments also demonstrated the spread of Mucor and Rhizopus during kilning (Haikara et al., 1977). They reported changes in the bacterial pattern during malting and identified Erwinia herbicola, Escherichia coli, Pseudomonas spp., Micrococcus spp., Bacillus spp. and actinomycetes at all stages of malting. Further laboratory studies revealed increases in bacterial numbers ranging from 5- to 36-fold (Follstad & Christensen, 1962; Flannigan et al., 1982; Kotheimer & Christensen, 1961), and in yeasts from 1.1-fold in stained and weathered barley and 5-fold in bright barley (Kotheimer & Christensen, 1961) to 200-fold in green malt (Haikara et al., 1977). Numbers of some moulds, such as Aureobasidium pullulans, G. candidum and Fusarium spp., may increase during germination (Flannigan et al., 1984), and then decline with kilning, but others such as Rhizopus spp. and Asp. fumigatus spread during kilning (Gyllang & Martinson, 1976b).

Micro-organisms of Beer

The antiseptic action of hops together with the poor nutrient status of beer, its low pH and the presence of ethanol, restricts the range of bacteria that can grow in finished beers (Priest, 1987). With regard to the Gram-positive organisms, lactic acid bacteria of the genera Lactobacillus and Pediococcus have been reported as the most common and dangerous spoilage agents, with some strains developing marked hop tolerance (Priest, 1987). In particular, Lactobacillus casei subsp. plantarum, L. coryneformis subsp. torquens, L. brevis and L. lindneri are associated with beer (Priest, 1987).

Some members of the family Micrococcaceae are known to survive in beer, and under certain conditions, Micrococcus kristinae will grow and cause spoilage (Priest, 1987). Members of the genus Bacillus, on rare occasions are known to cause problems in beer, but similarly to the micrococci, do not develop hop tolerance and do not pose serious problems (Priest, 1987).

Of all the pediococcal species, Pediococcus damnosus is the most common and feared species found in beer (Solberg & Claussen, 1973; Back, 1978; Lawrence & Priest, 1981). Indeed, this organism is apparently only found in beer, brewing yeast and wines, and not in brewing raw materials or plant materials. A recent comprehensive study of 840 strains from various sources, including wine, beer and brewery equipment, based on phenotypic tests and DNA sequence homology supported the integrity of six established species, including P. halophilus, P. parvulus, P. acidilactici, P. damnosus, P. dextrinicus and P. pentosaceus (Back, 1978; Back & Stackebrandt, 1978).

The presence of Gram-negative bacteria in the beer brewing process is generally regarded as being undesirable. Important Gram-negative spoilage bacteria include acetic acid bacteria (i.e. Acetobacter spp. and Gluconobacter spp.); Enterobacteriaceae representatives (eg. Enterobacter aerogenes, E. cloacae, E. agglomerans, Hafnia alvei, Citrobacter freundii, Klebsiella pneumoniae, K. oxytoca, Serratia spp., Proteus mirabilis and Obesumbacterium proteus (Thorwest, 1965; Bernstein et al., 1968; Von Riemann & Scheible, 1969; Prucha & Scheible, 1970; Anderson et al., 1971,; Eschenbecher & Ellenrieder, 1975; van Vuuren & Toerien, 1981; van Vuuren et al., 1981; McCaig & Morrison, 1984); Zymomonas, Pectinatus cerevisiiphilus and Megasphaera (van Vuuren, 1987). The occurrence of a few non-fermentative bacteria

has also been occasionally reported. These include Alcaligenes, Acinetobacter, Flavobacterium and Pseudomonas spp., which have been isolated from worts and brewing liquors (Masschelein, 1973; Eschenbecher & Ellenrieder, 1975; Priest, 1981). Spoilage of beer by these occasional contaminants has not been reported.

It is regarded as a good practice to test for the presence of undesirable yeasts at each stage of the brewing process (Rainbow, 1981; Hjortshoj, 1984). A list of yeast genera reported as brewery contaminants is given by Barnett et al. (1983). These include Brettanomyces, Candida, Debaryomyces, Dekera, Hanseniaspora, Hansenula, Kluyveromyces, Pichia and Saccharomyces.

Although direct contamination or growth of moulds in beer may not be common, their metabolic products do exert some influence on beer quality. Such moulds include Fusarium spp. (Prentice & Sloey, 1960), Alternaria spp., Stemphylium sp., Nigrospora sp. (Amaha et al., 1973; Yoshida et al., 1975), Asp. ochraceus, Asp. niger, Cladosporium sp. and R. arrhizus (Kneen, 1963).

Factors Promoting Microbial Contamination of Barley, Malt and Beer

Many factors affect the incidence of pests and diseases of barley and malt, and among these are sowing date, water content, temperature, insect infestations, aeration and condition of the grain.

In the field, sowing date plays a vital role in microbial attack of the barley plant. On both spring and winter cereals, there is usually more mildew (Erysiphe graminis) in late-sown than in early-sown crops (Last, 1957; Jenkyn, 1976). In spring-sown crops, infection by barley yellow dwarf virus occurs (Plumb, 1977). This invariably

reflects the climatic conditions.

Colonization of barley by micro-organisms has been studied during storage at different water contents (Hill & Lacey, 1983b). These workers reported that grain enters stores with a small inoculum of potentially damaging organisms whose subsequent development depends on the storage conditions. From the point of view of fungal growth, the moisture content controls the fungal species which grow. The temperature and, to a lesser extent, the oxygen:carbon dioxide ratio control the rate of growth and also help determine the species that grow (Hill & Lacey, 1983b, 1984). Such parameters as condition of the grain prior to storage, i.e. presence of damaged kernels, extent of already existing microbial contamination, etc., and the presence and activity of grain infesting mites and insects also affect the proliferation of the micro-organisms during storage (Christensen & Kaufmann, 1965, 1969; Christensen, 1973).

Pilot plant tests indicated that storage at 14.1% moisture content and 21° C did not result in any serious impairment of quality of the barley for malting and brewing, and may even have resulted in some improvement in certain quality factors (Follstad & Christensen, 1965). However, inaccurate measurement of moisture content may be responsible for unexpected cases of spoilage in commercially stored grain. Accurate measurement of moisture content, plus determination of numbers and kinds of storage fungi and of other biochemical properties may aid greatly in evaluating condition and storability of grain (Christensen & Linko, 1963).

The relationship between barley moisture content and invasion by storage moulds was studied by Tuite & Christensen (1955). In seed stored at a moisture content (M.C.)of 10 - 13% (wet weight) Aspergillus

and Penicillium remained static. At moisture contents of 13.8 - 14.2%, Asp. restrictus gradually invaded the germs. Also at a moisture content of 15 - 17%, species of the Asp. glaucus group such as Asp. repens, Asp. amstelodami, and Asp. ruber became the dominant flora.

In general, storage fungi grow most rapidly at temperatures of approximately 30° C (Christensen & Kaufmann, 1965, 1969) with a gradual decrease in growth commensurate with temperature declines (Christensen, 1973). However, Mulinge & Apinis (1969) reported that after self-heating had taken place, some micro-thermophilic species such as Asp. candidus and Asp. terreus, readily invaded the grain tissue of moist barley. At the other extreme of temperature, Burnell et al. (1966) reported some Penicillium species growing in moist barley stored at 3.8° C. Most storage fungi grow very slowly on cereal grains with 15 - 16% M.C. and their growth almost ceases at 5 - 8° C (Christensen & Kaufmann, 1965). Consequently, barley may be stored for several months below 5 - 10° C and at 15 - 16% M.C. (Papavizas & Christensen, 1958; Qasem & Christensen, 1958) without fungal growth. However, the resultant dormancy of barley kernels stored in this manner makes this method inappropriate for malting barley (Burrell, 1977).

In practice, however, the uniform M.C. and temperature may be difficult to arrive at in a bulk of stored grains. The development of a microbial succession culminating in massive proliferation of thermophilic organisms is usually associated with the faulty storage of undried feed barley (Clarke et al., 1967, 1969; Lacey, 1971; Clarke & Hill, 1981) and high temperature may be reached in dry-stored grain when 'hot spots' develop. Temperatures as high as 53° C have been recorded in hot spots within grain bulks during the Canadian winter (Wallace & Sinha, 1962) and in one hot spot a maximum of 64° C occurred

in May (Sinha & Wallace, 1965). The initiation of heating in this last case was attributed to the growth of Penicillium verrucosum var. cyclopium and P. funiculosum at -5 to 8° C during the winter, after harvest. Asp. flavus, Asp. versicolor, Absidia spp. and Streptomyces spp. succeeded the penicillia as the temperature increased. By their metabolic activity, micro-organisms generate water and heat, so they have the capacity to raise both the water activity and temperature of stored grain, a poor conductor of heat (Flannigan, 1987).

Most fungi grow well under aerobic conditons but absence of oxygen does not completely eliminate all fungi. Many bacteria and some yeasts are capable of anaerobic growth . Magan and Lacey (1984) reported that the growth of fungi decreased proportionally with decreasing oxygen concentrations down to <1% in the field, whereas the growth of some species of Aspergillus and Penicillium was only reduced at levels of oxygen <5%. However, an exception to this observation was P. roquefortii in which growth was actually stimulated at 5% oxygen. In moist barley, stored under conditions of low oxygen (0.5 to 1%), some yeasts (chiefly Candida and Hansenula anomala) formed the dominant flora in one of four phases of a succession of micro-organisms reported by Clarke et al. (1967).

The malting process offers an adequate environment for micro-organisms to proliferate. During steeping, the grain absorbs water, and releases soluble nutrients, and consequently dormant micro-organisms are activated: mould spores germinate, mycelium grows and yeasts and bacteria multiply. The steep liquor washes the superficial contaminants, a porportion of which are deposited on other kernels (Flannigan, 1987). Germination offers a period during which the

carbohydrate components of the grain are converted by the developing enzymes to fermentable sugars, which serve as substrate together with the nitrogeneous products for the micro-organisms to multiply. Flannigan (1987) reported that viable counts of bacteria and yeasts reached a maximum during germination of the barley, whether in laboratory or commercial malting.

Kilning has a profound effect on bacterial numbers. Follstad & Christensen (1962) reported that the very high number of bacteria on green malt was reduced to between 63% of the count for barley before steeping and 8-fold of that count. The combination of sulphuring (to produce a pale malt or to reduce nitrosamine formation) and kilning results in greater reductions in numbers of bacteria (Graff, 1972; Flannigan, 1983). In a laboratory experiment, Flannigan (1983) observed that the yeast number was also affected by sulphuring.

In general, mould numbers are also affected with kilning. Follstad & Christensen (1962) found that kilning brought about reductions in numbers of moulds on germinating kernels with the final counts being 63 - 70% of those for the original barley. However, as far as the percentage frequency of kernels contaminated by different moulds is concerned, Haikara *et al.* (1977) noted large increases with kilning for *Mucor* spp. and *Rhizopus* spp., and smaller increases for *Penicillium* spp., *Asp. glaucus* group and (from a very low level) *Cladosporium* spp. with no change for *A. alternata* and *Fusarium* spp.

The microbiological status of finished malt reaching a brewery or distillery will depend on its handling after production. Cross-contamination between, and also aerial contamination of, kernels may result during handling. In addition, malt usually picks up moisture in transit, and this encourages growth of micro-organisms. It was

observed that malt exported to Nigeria in sacks which became damaged showed greater levels of storage fungi than in the reference sample retained in the UK (Flannigan et al., 1984a).

Ideally, beer should remain free of micro-organisms. As has already been mentioned, the antiseptic action of hops, together with the poor nutrient status of beer, its low pH and the presence of ethanol, restricts the range of bacteria that can grow in finished beer (Priest, 1987). Introduction of new processes in brewing has brought with them new microbiological contaminants. In particular, the packaging of beers with very low oxygen content has led to the isolation of 'new' contaminants from beers. These include the anaerobic strains of Pectinatus cerevisiiphilus and Megasphaera (Lee et al., 1978). The various steps prior to hop-boiling are of little importance as a source of wild yeasts in the wort, insofar as none is sufficiently heat-resistant to survive boiling. Even in distillery practice, the yeasts of the malt, being predominantly aerobic, are unable to grow during the subsequent fermentation (Campbell, 1987). Wiles (1953) did, however, detect small numbers of fermentative yeasts in malt, hops and priming sugars.

Effect of Micro-organisms on the Malting Process

Moulds yeasts and bacteria are usually present within and beneath the seed coats of grain as well as on the surface of the kernels. The malting of barley provides an environment in which the nutrients of the barley kernel become increasingly available to micro-organisms. These are able to grow at temperatures commonly employed for malting. To maintain this association, a relationship between the physiology of the

micro-organisms and the barley, must be established. For example, enzymes secreted by the micro-organisms during the development of the kernels, during storage of the kernels after harvest, or during their subsequent germination may cause destruction of host tissue components (Brain, 1958). This subsequently makes nutrients available for the micro-organisms. The non-enzymic metabolic products of the microflora in the host tissues may also markedly affect the physiology of the host. However, substances inhibitory to microbial growth may also be produced by the host, otherwise the green malt would often be covered by microflora (Andersen et al., 1967). This is evident in papers dealing with antibiotic materials in plant tissue, for example in wheat and barley (Ark & Thompson, 1958).

Obvious examples of metabolic stimulators associated with micro-organisms and plants are the gibberellins (Stowe & Yamaki, 1959). Gibberellic acid is known to stimulate barley germination during malting, and is produced by some micro-organisms.

Data provided by Gilbert et al. (1954) suggest that during steeping and perhaps the malting process, some bacteria may produce substances inhibitory to the germination of barley and also may compete with barley for dissolved oxygen. Conversely, it is possible that some micro-organisms present during malting may be beneficial to the quality of malt.

The germinative capacity of the barley seed, so important for bringing about the required metabolic modification of the malt, is drastically reduced by microbial growth (Follstad & Christensen, 1962; Prentice & Sloey, 1960; Andersen et al., 1967). Increased availablity of oxygen in the grain has been related to more effective use of giberellins, which are known to induce germination (Banasik, 1969;

Sparrow, 1965). One explanation put forward to account for water sensitivity in barley is the presence in the lemma, palea and pericarp-testa of large numbers of field organisms which compete for available oxygen during steeping. When water sensitive barley is placed in an excess of water, the limitation of entry of oxygen to the embryo results in the kernel either failing to germinate or germinating slowly. Under these circumstances, some micro-organisms are able to invade the embryo, and the kernel loses its viability (Flannigan, 1987). Some experiments on field germination with Gliocladium roseum, a saprophytic mould which had been isolated from barley (Pepper & Kiesling, 1963; Lynch & Prynn, 1977) noted that the effects of low oxygen availabity on germination and viability were exacerbated by the presence of fungi. In further experiments, Harper & Lynch (1981) observed that this organism grew at the embryo end of the kernel, i.e. at the point of entry of oxygen. Earlier, Harper & Lynch (1979) had demonstrated that the nitrogen-fixing bacterium Azotobacter chroococcum could inhibit germination by competing for oxygen; its effects being greater at low oxygen concentrations.

In an experiment in which barley kernels were contaminated with Fusarium spp. during steeping, Sloey & Prentice (1962) noted that single isolates of F. bulbigenum var. lycopersici (= F. oxysporum f. sp. lycopersici), F. graminearum, F. nivale and an unidentified species caused significant reductions in rootlet growth.

In special germination tests to assess possible effects of Helminthosporium sativum (= Cochliobolus sativus) and Fusarium spp. on malting, badly infected kernels from Iowa and southern Minnesota were set to germinate on moist blotting paper at 16° C. Only a little over 67% germinated, and of these , more than 25% showed a stunted, rotted

or aborted primary rootlet and a contorted or very short acrospire. C. sativus was more destructive than Fusarium spp. (Christensen & Stakman, 1935). Perhaps this severity in the destruction may be explained by the fact that there is a chemotropic relationship between the roots of barley and the pathogen, C. sativus. It was observed in a laboratory experiment that the same organism grew chemotropically towards the sterilized barley roots or their exudates (Janson et al., 1988).

Gyllang et al. (1977) in their laboratory malting experiments found that the growth of Asp. amstelodami, Asp. fumigatus and Rhizopus oryzae during germination of barley increased extract yield, total soluble wort nitrogen, α-amino nitrogen and raised the attenuation limit in malt obtained from such barley.

Kilning generally results in a reduction in microbial populations, particularly if sulphuring is applied to counteract the formation of nitrosamines (Flannigan, 1983). However, the final populations are substantial, and include fungi, yeasts, bacteria and thermophilic actinomycetes (Haikara et al., 1977; Flannigan, 1982, 1984).

Effects of Micro-organisms on Barley, Malt and Beer Quality

The presence of micro-organisms on or within the grain may produce both physical and biochemical changes to the grain. These changes may be beneficial or lethal depending on who uses the grain and for what purpose. The presence of moulds, yeasts or bacteria in the grain may lead to the discolouration or damage of the embryos of the grain. This reduces the germinative capacity, which is important to the maltsters as well as the farmers. Alternatively, the grain may be rendered unsightly thus reducing its market value. The biochemical reactions in

malt may lead to the production of toxic materials that constitute a health hazard for man and his animals (Christensen & Kaufmann, 1965).

Effects on barley

Reduction in germination:

Moulds are the most studied micro-organisms, as pathogens of barley. In the field, seedling blight and root rot caused by Fusarium spp. and Cochliobolus sativus are well known. Thus, Jorgensen (1983) noted that F. nivale, C. sativus, F. avenaceum, F. culmorum, F. graminearum were, together with H. gramineum (= Pyrenophora graminea), the most important seed-borne pathogens of barley in Denmark. Tuite & Christensen (1955) stored barley at 13.7 - 16.6% M.C. for 7 months at room temperature, and found a close correlation between an increase in storage fungi and a decrease in percentage germination of the seed. Similarly, Follstad & Christensen (1962) reported that losses in germination percentage of barley were found to accompany increases in the numbers of storage fungi (mainly members of the Asp. glaucus group). Furthermore, Lund et al. (1971) determined that the germination capacity of barley stored in experimental silos at 16 - 17% M.C. diminished on storage, with an accompanying growth of members of the Asp. glaucus group, whereas germination of samples stored at 18 - 20% M.C. was so slight that they could not be malted. Again, Hill & Lacey (1983b) noted that germination of barley correlated with water activity of storage (for 6 - 9 months) and with the growth of storage fungi. It has been found that Aspergillus species invade the germ exclusively and preferentially (Tuite & Christensen, 1952, 1955; Qasem & Christensen, 1958; Papavizas & Christensen, 1958, 1960). However, some researchers have indicated that a wide variety of fungi, bacteria

and viruses exist within apparently healthy plants, but not all of these microbes cause disease at a later period (Simmonds, 1941; Hayward, 1974; Boss, 1978).

Harrison & Perry (1976) studied the mechanisms of barley seed deterioration. They concluded that Asp. repens, P. cyclopium and Fusarium culmorum became dominant within certain moisture ranges. Conversely, Christensen (1973) considered that storage fungi were the primary cause of deterioration. Yet, Roberts (1973) regarded deterioration as occurring independently of micro-organisms, but nevertheless following a defined sequence of ultrastructural and physiological changes.

Formation of hot spots is yet another influence brought about by micro-organisms.

Production of enzymes

Since the invading micro-organisms utilize the grain as a source of nutrients, chemical deterioration often ensues. The production of extracellular enzymes leads to the degradation of the starchy, proteinous and fatty components of the grain. Because of the high lipolytic activity of moulds, their growth is accompanied by an increase in fatty acids (Christensen et al., 1949; Hummel et al., 1954). Lipid hydrolysis takes place much more rapidly than protein or carbohydrate degradation in stored grain. This led to the introduction of the fatty acid value by cereal chemists as an index of mould deterioration in stored grains (Pomeranz, 1982). However, it was found by Bottomley et al. (1952) that moulds differed in their ability to alter the fat acidity of maize. These workers found that members of the Asp. glaucus group caused only a slight increase in acidity whereas

Asp. candidus, Asp. flavus, Penicillium spp. and Fusarium spp. caused marked increases. In addition, McGee & Christensen (1970) emphasised that the fatty acid value was not helpful in determining the early invasion of barley seeds by storage moulds. Two widely occurring leaf surface yeasts, Cryptococcus laurentii and Rhodotorula glutinis, were shown by the titration method (Ruinen, 1966) and by spectrophotometry (Heinen & de Vries, 1966) to cause some hydrolysis of cutin.

With regard to the conversion of carbohydrates to simple sugars, Lund et al. (1971) found a good correlation between increases in mould growth and reducing sugars and decreases in non-reducing sugars in stored barley. This property has been very useful to brewers who apply the microbial enzymes to reduce the problems in brewing such as beer filtration rate, wort separation, gel and haze formation which are associated with the presence of β-glucan, a cell wall polysaccharide.

Effects on malt and beer

As previously stated, only a few groups of micro-organisms actually grow in beer and therefore lead to spoilage. This reflects the low pH and redox potential, the content of inhibitory products derived from yeast (ethanol, fusel alcohols and esters) and, to a lesser degree, to its content of hop antiseptics (Rainbow, 1979). Although Rainbow (1979) noted that beer spoilage organisms included bacteria and yeasts, but not moulds, other workers reported cases of fungal involvement in beer deterioration.

Barley contaminated with micro-organisms shows poor germination, and produces malt with characteristics which may be beneficial or undesirable. Kneen (1963) found that beers derived from weathered

barley (both naturally weathered in the field and 'artificially' weathered during laboratory malting) showed high protein modification, reduced gas stability and a greater formation of off-flavours. On applying pure cultures to barley after draining of the last steep liquor, Kneen (1963) observed that some fungi exerted minor effects whereas others, including Asp. ochraceus, Asp. niger, Rhizopus arrhizus and Fusarium spp. produced a marked detrimental effect on beer flavour and haze stability. This worker postulated that metabolic products of the micro-organism, found in the husk, and carried on the malt, inhibited the action of papain, a common stabilising agent used in brewing. Gyllang et al. (1977) reported that beer brewed with malt contaminated with R. oryzae was distinctive without any special off-flavour. Yet, Asp. fumigatus gave a pronounced roughness and a staling flavour. Asp. fumigatus and R. oryzae have also been found to increase α-amino nitrogen and soluble nitrogen in beer (Gyllang et al., 1977).

Beer colour was also affected by the presence of moulds. Kneen (1963) reported that malt prepared from weathered barley produced beer with a high colour, as was the case when malt prepared after inoculation with Asp. niger, Cephalothecium sp. (= Trichothecium), Rhizopus sp. and especially Fusarium sp. or R. arrhizus was used. In subsequent work, increased colour was noted when Asp. fumigatus, R. arrhizus, F. culmorum and F. avenaceum (Gyllang et al., 1977; Haikara, 1983) were present. In reviewing the effects of micro-organisms of barley on malt and beer properties, Etchevers et al. (1977) noted there were pronounced changes in the characteristics of malts prepared after storage of the barley for one month. The extract and wort nitrogen levels were higher, but α-amylase and diastatic power were much lower. However, Prentice & Sloey (1960) carried out experiments in which they

inoculated steeped barley in a micro-malting system with 97 different micro-organisms including bacteria, moulds and yeasts. Most of these had been previously isolated from barley. These workers found that Fusarium spp. affected malt characteristics, notably increased diastatic power and α-amylase activity, increased soluble wort nitrogen and decreased gas stability in beer. Fleming et al. (1961) showed that up to 15% of the total α-amylase activity in a malted, mouldy wheat sample was of fungal origin. They also indicated that very little bacterial amylase could be detected in malted wheat. Further work on Fusarium spp. by Sloey & Prentice (1962) indicated a general tendency towards increased steep and respiratory loss, with a concomitant reduction in malt recovery. There were also higher α-amylase and extract values.

Gushing or reduced gas stability is a well known problem in brewing industries and many researchers have delved into finding the cause and solution to the problem. The results of the studies by the Malt Research Institute (1955) indicated that weathering of barley was the cause of gushing in beer. It has since been demonstrated that malt made with field inoculated barley can give rise to gushing (Haikara, 1983). The formation of gushing in beer as caused by micro-organisms, e.g. Fusarium spp., has been highlighted by Kneen (1963). Prentice & Sloey (1960), Sloey & Prentice (1962) and Andersen et al. (1967). Gjertsen et al. (1965) observed a strong gushing tendency in beers made from stored barley of high moisture content. According to their results, gushing can be divided into two types, namely 'primary gushing', which occurs periodically and appears to be related to the quality of the malt (microflora), and 'secondary gushing', which is due to faulty production processes or to improper treatment of the bottled

beer. These workers indicated that a gushing inducing factor could be extracted from malt and that the real cause of primary gushing was the presence of specific substances. Gjertsen and co-workers demonstrated experimentally that an interaction between the growing mycelium of Fusarium and the germinating barley is required to cause gushing. Amaha et al. (1973) isolated and purified a polypeptide (composed of 14 amino acids) gushing factor, produced by Rhizopus sp.. This crystalline substance was capable of inducing vigorous gushing, even at 0.05 mg l^{-1}, in normal beer. These workers also isolated gushing-inducing factors from Stemphylium and Fusarium species. The Stemphylium factor was characterized as a peptodiglycan, whereas the corresponding compounds from Fusarium were polypeptides. An investigation of malt from nine malting plants in Scandinavian countries and England (Gyllang & Martinson, 1976a) showed that malt which caused gushing in beer contained high proportions of grains contaminated by Aspergillus, Rhizopus and Penicillium. The most common species included Asp. amstelodami and Asp. fumigatus. In a further study (Gyllang & Martinson, 1976b), these two moulds were shown to cause gushing in beers when they were inoculated in the steep water during malting. Although Amaha et al. (1973) concluded that elimination of mould-infected barley and malt is the most reliable and effective way to prevent gushing in breweries, it is not always easy to carry out. This is due to the presence of fungal propagules.

Besides the implication of micro-organisms in gushing, other substances (non-living) have been reported to cause problems in breweries. One notable example is barley β-glucan. Indeed, this high molecular weight polysaccharide is reported to cause problems in wort separation, beer filtration, gel and haze formation and reduction in

extract yield (Bamforth, 1982).

The modification of barley corns from being hard and vitreous to being friable and mealy, which occurs during germination, is due in large part to the dissolution of the cell walls of the starchy endosperm. The process is effected by a series of enzymes which act to render the food reserves accessible to other enzymes which will hydrolyse them. Three different categories of endo-β-glucanase in malt have been described: (1) Endo-1-3-β-glucanase, (2) Endo-1-4-β-glucanase and (3) endo-barley-β-glucanase. The actual significance of each of these enzymes to the degradation of barley β-glucan is not known (Bamforth, 1982). Endo-1-4-β-glucanase is largely confined to the husk, where its role might be to degrade cellulose (Ballance et al., 1976). Although grain undoubtedly contains some Endo-1-4-β-glucanase, it is known that much of this activity can originate from micro-organisms, which contaminate barley (Hoy et al., 1981). Endo-1-3-β-glucanase has no action on the β-glucan which can be extracted from flaked barley but it will reduce the viscosity of the β-glucan isolated from 48 h green malt (Bathgate et al., 1974). The cells, which are directly beneath the aleurone, have walls much thicker than those of the central endosperm and these walls are rich in 1-3-β-glucan (Fulcher et al., 1971). Endo-1-3-β-glucanase may have a role in digesting these walls. Probably the only glucanase in malt which has a role in digesting the mixed linkage β-glucan is the endo-barley-β-glucanase. This enzyme is either absent or, at most barely present in raw or steeped barley (Luchsinger et al., 1958). Endo-barley-β-glucanase is heat-labile and, without due caution, is totally destroyed during kilning. It has been reported that 33% of the β-glucanase survives mashing at 65° C, but it is likely that the residual glucanolytic activity is due to solubilase

(Bamforth & Martin, 1981).

Wort separation rates, extract yields and beer filtration may be improved by using commercial β-glucanases of bacterial or fungal origin in the mash tun (Stentebjerg-Olesen, 1980; Enkenlund, 1972). These enzymes are more resistant to heating than are the β-glucanases in malt. Particularly efficient is the enzyme complex of the fungus Trichoderma (Stentebjerg-Olesen, 1980). This organism can be grown on brewer's grains and, in so doing, it produces β-glucanase (Bamforth & Martin, 1981). The Trichoderma enzymes increase the rate of wort separation from all malt mashes as well as from mashes which include adjunct (Bamforth & Martin, 1981; Stentebjerg-Olesen, 1980). There have been two reports of the use of cellulase preparations from the fungus Trichoderma for the complete conversion of barley β-glucan to glucose (Prentice et al., 1980; Martin & Bamforth, 1981). Subsequent experiments indicated that this method based on Trichoderma may be used for malts (Martin & Bamforth, 1981), worts and beers (Bamforth & Martin, 1981). On a laboratory scale, microbial β-glucanases have been immobilized and used to decrease the viscosity of wort and to increase the filtrability of beer (Linko & Linko, 1979). However, it has been found that immobilization of endo-β-glucanases may change their mode of action to an exo-form, in which case they are much less effective in reducing viscosity (Svensson & Ottesen, 1978). Although Brunswick et al. (1987) have reported recently that two, namely 1,3;1,4-β-D-glucanase isoenzymes may develop in response to application of gibberellic acid during germination of barley, the microbial β-glucanases may be preferred since they are more heat stable than barley or malt β-glucanases.

There are two objections to a high bacterial count in the mash.

Firstly, the presence of even moderate numbers of bacteria in the original mash means there will be a much larger number present after one or two days of fermentation at 24° C to 32° C. These grow at the expense of some of the carbohydrates present, thereby lowering the yield of alcohol. Secondly, the production of objectionable bacterial fermentation products adversely affects the quality of the distillate (Boruff et al., 1938) or beer. Some strains of Lactobacillus were particularly deleterious to fermentation and were responsible for reducing the final pH, increasing final gravity and reducing the alcohol concentration in fermented wash in Scotch whisky fermentation (Barbour & Priest, 1988). Acetic acid bacteria oxidise ethanol to acetic acid, and the infected beer may become 'ropy' (Rainbow, 1979). Spoilage of beer by Pediococcus cerevisiae (P. damnosus) is characterized by acidity and diacetyl formation, while some capsule-forming strains turn beer ropy (Rainbow, 1979). Zymomonas anaerobia converts glucose and fructose largely to ethanol and CO_2, with simultaneous production of acetaldehyde. This latter product, together with H_2S, causes the distinguishing odour and flavour (rotting apple) of beers spoiled by this organism (Rainbow, 1979). Again, Rainbow (1979) noted that all beers containing a residium of fermentable sugar were open to attack by yeasts, causing 'frets' and off-flavours, or sometimes over attenuation. Such yeasts include those belonging to asporogenous groups, i.e. Candida, Brettanomyces, Kloeckera, Cryptococcus, Torulopsis and Rhodotorula, and to sporogenous genera, namely Pichia, Hansenula, Hanseniaspora and most important of all, Saccharomyces, of which the super-attenuating S. diastaticus is an example. Rainbow (1979) noted that spoilage strains occurred even within the species S. cerevisiae and S. uvarum to which culture ale

and lager yeasts belong, respectively. For example, S. cerevisiae var. ellipsoideus was reported to cause haze. As with bacteria, the yeast contaminants during fermentation multiply at the expense of the culture yeast (Rainbow, 1981). Maule & Thomas (1973) reported a detailed case history of an outbreak of 'killer' yeast infection replacing the culture yeast and producing an objectionable off-flavour, in addition to poor attenuation and other defects. 'Killer' yeasts are an extreme form of contaminant, killing and replacing a sensitive culture yeast by the zymocins they so produce.

Health Hazards Associated with Micro-organisms in Barley and Malt Production

Although not all micro-organisms present in barley kernels have the potential of growing and causing diseases, some organisms cause great concern. The potentially harmful effects of seed-borne micro-organisms on man and livestock may be discussed under the topics of respiratory hazards, infections and mycotoxins.

Respiratory hazards

It has been known that inhalation of high concentrations of grain dust over long periods may lead to some health hazards to individuals working in the grain industry. Grain dust consists of particles/fragments and end products of grain and chaff, soil fragments, bacteria, yeasts, spores and mycelium of moulds and actinomycetes, mites, and insects. Lacey (1975) reported that workers handling grain may become sensitized to micro-organisms, and develop

allergic conditions. Airborne spores may cause two types of allergy: immediate allergy and delayed allergy. Immediate allergy results from type I reactions, ie. the response is evident more or less immediately after exposure to the allergen or antigen, and the effects last only a few hours. This reaction is associated with inhaling spores of certain fungi and actinomycetes. After inhalation, spores which are larger than 10 μm in diameter are largely deposited on the mucus membranes of the naso-pharynx. Any allergic reaction caused is termed rhinitis or hay fever. Spores in the range of 4 - 10 μm, mainly deposited in the bronchi and larger bronchioles, provoke an asthmatic type of response in which there is difficulty in breathing (dysponoea). The type I reaction is characteristic of 10% of the population, who are atopic. Such individuals are usually allergic to a wide range of allergens and react to spore concentrations which do not normally affect the remainder of the population, ie. 10^4 - 10^6 spores m^{-3}. Delayed allergy results from type III reactions and usually occurs when the non-atopic individual is exposed to high concentrations ($>10^6$ spores m^{-3}) of a particular type of spore containing a single specific allergen. Unlike type I reactions, it does not develop for about 4 h and may continue for >48 h. It mediates by precipitating antibodies, resulting in tissue damage. The spores, which are <4 μm in diameter, stick on the respiratory surface of the terminal bronchioles and air sacs of the alveoli. If the spores induce an allergic response, the condition is described as extrinsic allergic alveolitis. In the lung, there is a perivascular inflammatory response. The overt symptoms are malaise, fever, a dry cough and breathlessness. With repeated attacks, the lung may be permanently damaged.

The types of spores associated with barley which are most likely

to induce immediate allergy include the common field fungi Alternaria alternata and Cladosporium herbarum, with Epicoccum purpurascens causing rhinitis only. The delayed allergy may be caused by spores of the thermophilic actinomycetes, Thermoactinomyces vulgaris and Micropolyspora faeni (Pepys et al., 1963) and the moulds Aspergillus fumigatus, Asp. clavatus (Riddle et al., 1968) and Penicillium spp. These fungi are associated with self-heated barley and fodders (Lacey, 1975) and are the causal agents of farmer's lung.

Maltworker's lung (Riddle et al., 1968) differs from other chronic respiratory diseases, such as bronchitis and emphysema, the incidence and severity of which have been noted in the malting industry. This extrinsic allergic alveolitis is now recognised as an occupational disease, like farmer's lung. It is caused by Asp. clavatus. Once Asp. clavatus has been established in floor maltings it may be seen sporing profusely on the surface of the kernels. Consequently, clouds of spores are released into the atmosphere during the turning of green malt (Riddle et al., 1968). As Asp. clavatus is rarely found in dried barley, the primary source of contamination of malting premises remains uncertain. Pigeons, which have access to the grain store have been implicated in one maltings (Riddle et al., 1968). However, this organism has been found in harvested barley in Canada (Wallace, 1973), Egypt (Abdel-Kader et al., 1979) and Rumania (Stankushev, 1969).

Infection

The tissues (other than in the lungs) of man and animals may be invaded by micro-organisms, giving rise to mycoses. Asp. fumigatus, as well as being allergenic, is well known as an opportunistic pathogen,

causing respiratory infections in man and animals. The organism also causes mycotic abortions in cattle (Austwick, 1972). Asp. flavus, Asp. nidulus and Asp. niger have also been occasionally implicated as pathogens. Absidia spp. and Mucor pusillus may cause phycomycosis and gastric ulceration and Absidia spp. also are often reported in mycotic abortion (Ainsworth & Austwick, 1973). Most of the fungi and actinomycetes involved in health hazards have been found on dried barley grain (Flannigan, 1969), malt (Gyllang & Martinson, 1976b) and in moist barley in 'sealed' silo storage (Clarke & Hill, 1984). Indeed, these organisms, if allowed to grow in barley used for malting or feed, may pose health problems to man and livestock.

Mycotoxicoses

Mycotoxicosis is the poisoning of animals and man by feed and food products contaminated by toxin-producing fungi. Some of the toxigenic fungi are common and widespread.

Asp. clavatus is associated with malt production, and is toxigenic. The mycotoxins produced include two tremorgenic compounds, ie. tryptoquivaline and tryptoquivalone (Glinsukon et al., 1974; Clardy et al., 1975). Although the effect on humans is not known, pulmonary mycotoxicosis and tumour development have been observed in experimental mice inoculated nasally with spores (Blyth & Hardy, 1982). However, the possibility of stock fed on by-products contaminated with this organism developing unhealthy conditions should not be ruled out. Blyth et al. (1977) were able to isolate the mould from screened-off culms and rootlets. Fatal tremorgenic disease has been reported among cattle fed upon sorghum malt residues, which were contaminated with

Asp. clavatus (Kellerman et al., 1976). The danger of aflatoxin was recently emphasised by Dutton (1988). In particular, aflatoxin B, the major member of the aflatoxin group, is the most potent naturally occurring carcinogen known, and is mutagenic and teratogenic, as well as an acute toxin. Aflatoxins, produced by Asp. parasiticus and Asp. flavus, are the best known of the mycotoxins, and have been implicated in hepatitis, and the death of more than 100 people in Western India (Krishnamachari et al., 1975). Such aflatoxins were the cause of the death of 100,000 turkey poults in England (Sargeant et al., 1961). Asp. flavus grows and produces aflatoxins on several agricultural products such as wheat, rice, maize and sorghum, given appropriate conditions of aeration, temperature and moisture (Hesseltine et al., 1966). Scott et al. (1970) reported that aflatoxins occur naturally in the sprouts of barley and malt. However, at present there is little evidence that significant quantities of mycotoxins pass through into beer. However, it was found that a little aflatoxin B_1, citrinin or ochratoxin A, which had been added at various stages during the malting and brewing processes, could be detected in beer (Gjertsen et al., 1973; Krogh et al., 1974; Chu et al., 1975; Nip et al., 1975). Citrinin, produced by Asp. ochraceus and P. citrinin, has been detected in barley and oats (Scott et al., 1970). Ochratoxin is produced by Asp. ochraceus and some other fungi and as much as 28 -39% of the ochratoxin A present may remain in spent grains (Nip et al., 1975).

Other mycotoxins include trichothecenes, such as T-2 toxin , and zearalenone or F-2 toxin, which are produced by Fusarium species, found on cereals. T-2 toxin solutions of specified (Flannigan et al., 1985) and unspecified (Nummi et al., 1975) concentrations depress germination of barley and α-amylase activity in malt prepared from

spiked barley, and reduced ∝-amino nitrogen levels. Such toxins affect the growth of brewing yeast (Flannigan et al., 1985, 1986) Mannio & Enari (1973) observed that no T-2 toxin or zearalenone appeared to pass from malt prepared from barley heavily contaminated with F. culmorum into beer. However, up to 4.6 mg l^{-1} zearalenone was found in some home-brewed opaque maize beers in Zambia (Lovelace & Nyathi, 1977). Failure to detect mycotoxins in malt or beer may therefore be interpreted to mean that they are either inactivated or degraded. Nip et al. (1975) noted that 8 - 20% of the ochratoxins originally present during fermentation could be recovered from the yeast. Although mycotoxins may have an effect on yeast growth (Schappert & Khachatourians, 1983, 1984; Flannigan et al., 1985) it is noted that yeast has some capacity for detoxification. Stinson et al. (1978) found that patulin disappeared during fermentation, and Flannigan et al. (1984b) found that, in the presence of the trichothecenes T-2 toxin and diacetoxyscirpenol, attenuation proceeded as normal after an initial lag. However, Mannio & Enari (1973) noted that Fusarium toxins were not completely inactivated during beer production; a part remaining in the spent grains. The growth of toxigenic organisms should be discouraged in grain or beer because the degraded products may also be toxigenic. However, in practice, the grain would have to be highly contaminated for mycotoxins to accumulate at lethal levels (Krogh et al., 1974), and barley of such quality would never be malted (Andersen et al., 1967).

A knowledge of the nature, magnitude and distribution, and the role of microbial populations in (commercial) malting barley ecosystems

remains incomplete. Most of the published work on the microbiology of malting has been concerned with laboratory micro- or pilot-maltings which simulate conditions of those in commercial maltings (Sheneman & Hollenbeck, 1960; Kotheimer & Christensen, 1961; Follstad & Christensen, 1962; Haikara et al., 1977). These authors were concerned mainly with the changes in numbers of micro-organisms during malting. Very little research work has been reported on the microbiology of commercial malting. However, Anderson et al. (1967), Gyllang & Martinson (1976b), Flannigan et al. (1984) and Douglas & Flannigan (1988) have published limited data on limited aspects of commercial malting. It is clear that some differences exist between the microbiology of malting barley on a laboratory as compared to a commercial scale. Thus, Flannigan et al. (1982) noted marked differences in the development of microbial populations, during malting on a laboratory scale compared with malting on a commercial scale.

OBJECTIVES

Against the limited background of previous work, this study reports a detailed investigation of the microbiology in commerical malting. Emphasis has been placed on bacterial populations at different stages of malting, viz. dried barley, steeped grain and liquor, kilned- and screened malts. Because the ultimate aim of a maltster is to produce a well modified malt, the study included an investigation of the possible involvement of micro-organisms in the quality of finished malt. This aspect was considered in two ways. Firstly, the effects of representative groups of micro-organisms on malt characteristics was examined in laboratory experiments. Secondly,

the effects of extracellular enzymes produced by some of the isolated micro-organisms on the carbohydrate components of the barley were considered.

MATERIALS AND METHODS

CHARACTERIZATION OF MICRO-ORGANISMS ISOLATED FROM MALTING BARLEY

Collection of Samples

Samples were collected from a single production run of malt at a commercial maltings in the south of Scotland. The samples included dried barley seed (Hordeum vulgare cv. Triumph, 1985 English crop) collected from a storage silo; "first" and "second" steeped barley corn collected from the exit at the bottom of the steep tank (this conveyed the grain to the saladin box, after draining the steep water); green malt at the end of a 5 - day germination period; sulphured (180 mg kg^{-1}) kilned malt collected from the conveyance duct leading to the screens, and screened malt. Samples of first and second steep water, and water freshly obtained from the bore hole were also examined. All samples were collected following Institute of Brewing (IOB) recommended methods of analysis (Wood, 1986). A flow diagram of the malting processes and the exact locations of sampling points have been included in Fig. 1.

The primary grain samples were collected with a clean aluminium shovel and placed into a sealable polyethylene bag to give an approximately 4 kg bulk sample. The green malt samples were collected at 14 points from the surface, and from the depth of about 50 cm along the length of the saladin box. For running samples (kilned malt and steeped grain), the kernels were collected directly into the polyethylene bag. Water samples were collected in sterile 500 ml stoppered glass bottles. Where possible, samples were collected from several locations and mixed prior to microbiological examination.

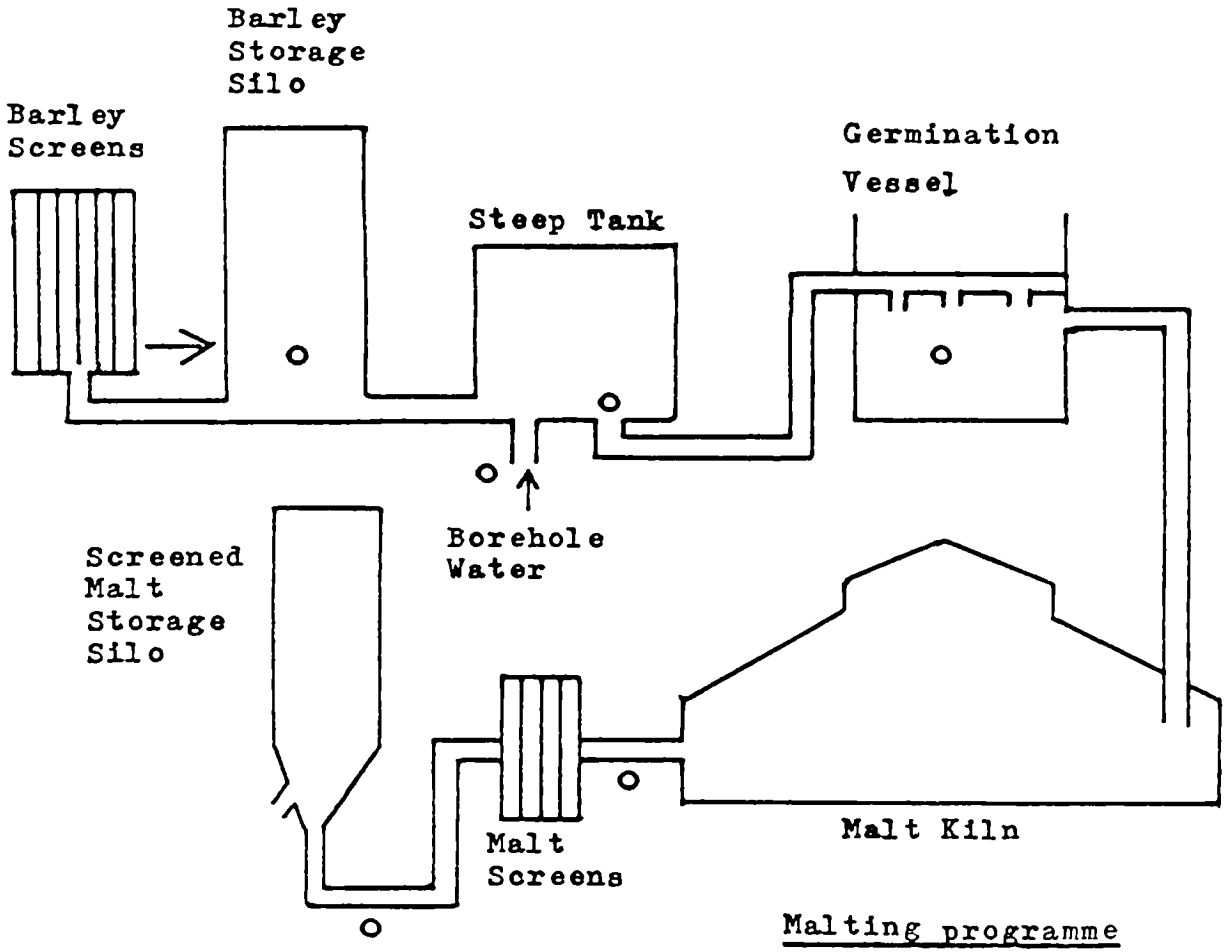


Figure 1. Flow diagram of the commercial malting in Southern Scotland

Microbiological Examination

Analyses were commenced within an hour of collection and sterile precautions were used where appropriate. Four categories of micro-organisms were determined, namely moulds, yeasts, aerobic heterotrophic bacteria and lactobacilli.

Media and incubation conditions

Media:

Potato dextrose agar (PDA; Oxoid) was used to enumerate both the filamentous fungi and yeasts. The PDA was supplemented with penicillin G (5 IU ml^{-1}) and streptomycin sulphate (0.14 mg ml^{-1}) to suppress bacterial growth. In addition to PDA, the proportions of barley kernels contaminated with filamentous fungi, were evaluated on a range of other media. These included oatmeal agar (OMA; Difco) supplemented with 0.2% (w/v) pentachloronitrobenzene (PCNB) in toluene, 10% (v/v) Triton X-100 and 0.01% (w/v) chlortetracycline, modified PDA (mPDA) amended with 38 mg l^{-1} neomycin and 0.2% (w/v) PCNB (Moll, 1981), a modification of Spenzieller Nährstoffarmer agar (mSNA; Nirenberg, 1976), i.e. a low nutrient agar amended with 0.2% (w/v) PCNB, 0.01% (w/v) chlortetracycline, 0.01% (w/v) chloramphenicol; malt salt agar (MSA) prepared by adding 10% (w/v) sodium chloride to malt extract agar (Oxoid). Plate count agar (PCA; Oxoid) otherwise known as glucose yeast extract agar (GYEA) and the selective medium of de Man, Rogosa and Sharpe (1960) (MRS; Oxoid) were used to determine the counts of aerobic heterotrophic bacteria and lactic acid bacteria (lactobacilli and streptococci) respectively. Cycloheximide (otherwise known as actidione, 5 mg ml^{-1}) was incorporated into PCA and MRS agar to suppress the growth of fungi. The MRS agar was modified by adding

0.5% (w/v) each of maltose and sucrose, and vitamins. The vitamin solution was made up of thiamine hydrochloride(10 $\mu g\ ml^{-1}$), biotin (10 $\mu g\ ml^{-1}$), folic acid (500 $\mu g\ ml^{-1}$), calcium pantothenate (500 $\mu g\ ml^{-1}$), riboflavin (2500 $\mu g\ ml^{-1}$) and pyridoxine (500 $\mu g\ ml^{-1}$); and added at the rate of 1 ml per 100 ml of medium. Each medium was prepared according to the manufacturer's specifications.

Incubation conditions:

For initial analysis of barley collected during malting, all plates were incubated at 25° C for 7 - 14 days. However, in subsequent investigations of dried barley and screened malt for assessing filamentous fungal taxa, inoculated plates were incubated at 25° C for 10 days. Inoculated media were incubated at 4°, 10°, 15° C for 14 days for the recovery of psychrophiles, at 25°, 30° and 37° C for 4 - 7 days for the growth of mesophiles; and at 50° and 60° C for 2 days for the isolation of thermophiles. The nucleopore filter- containing plates were incubated at 25° C for the recovery of possible micro-organisms for 2 days. Conversely, MacConkey agar plates were incubated at 30° and 44.5° C for 2 days in order to enumerate total coliforms and faecal coliforms, respectively.

MICROBIOLOGICAL METHODS

Methods used for the examination of microbiological populations in the samples included microscopy, direct and dilution plating techniques and membrane filtration.

The Dilution Plate Technique

For direct comparison (Bottomley et al., 1952) 100 kernels from each grain or malt sample were analysed. The grains were placed in an ethanol-flame-sterilized vortex beaker of an MSE top-drive homogeniser, containing 50 ml sterile 0.12% (w/v) water agar (No. 1; Oxoid), and homogenised for 10 min at 14,000 rpm. The homogenate was adjusted to 100 ml with further water agar, mixed thoroughly, and 10-fold dilutions prepared to 10^{-6} (Flannigan, 1977). Dilutions of steep and bore-hole water were also made to 10^{-6} using water agar as a diluent. Aliquots (0.1 ml) were pipetted and spread onto triplicate plates of media. The media used included PCA, PDA, MRS and MacConkey agar (Oxoid).

Direct Plating Technique

One hundred kernels were placed directly onto a set of ten 9.0 cm diameter Petri dishes containing PDA, MSA, mPDA, mSMA or OMA in order to ascertain the proportions of grains carrying different filamentous fungi.

Membrane Filtration Technique

Since the level of microbial contaminants, if any, in bore-hole water is usually very low, a membrane filtration technique was carried out to determine the microbiological contaminants. Aliquots (100 ml) of the bore-hole water were filtered (0.45 um porosity Nucleopore filters). These filters were applied to the surface of plates of PDA, PCA, MRS and MacConkey agar.

Electron Microscopy

This was carried out by a modified method of Mudarris & Austin

(1988). Barley and malt kernels, collected from the stages of commercial malt production, were fixed overnight at 4° C in 5% (v v glutaraldehyde buffered with river water (pH 7.0). Samples were dehydrated by immersion for 15 min periods in graded acetone solutions (25, 50, 75, 90 and 100% (v/v), critical-point dried, mounted with Araldite on aluminium stubs, sputter-coated with gold (Polaron sputter coater), and examined in a Cambridge Stereoscan scanning electron microscope.

Enumeration of Micro-organisms

For bacterial and yeast counts, the dilution plates containing between 30 and 300 colonies were counted (Flannigan, 1977 , whereas the number of filamentous fungi was determined from PDA supporting the growth of 5 to 50 colonies (James & Sutherland, 1939). Scores higher than 300 colonies were recorded in the case of Nucleopore filter-containing plates. These procedures were carried out on triplicate plates.

The percentage of kernels bearing different filamentous fungi were recorded from direct plates and used to compute the mould frequency indices (MFI's) (Flannigan, 1982) as:

$$MFI = \frac{\text{Sum of maximum recorded \% contamination of kernel by different moulds*}}{100}$$

*where several media are used MFI indicates the mean number of mould types per kernel

TAXONOMY OF THE ISOLATES

Isolation, Purification and Maintenance of Isolates

From the dilution plates, 400 colonies of aerobic heterotrophic bacteria (50 from each sample type), 30 colonies of presumptive lactobacilli, 50 colonies of yeasts and 96 colonies of filamentous fungi (including 47 colonies from the direct plating technique) were subcultured using a technique (Grid system) which ensured random selection. The organisms were streaked onto fresh media, and incubated at 25° C for 7 days. Pure cultures were subsequently stained using Hucker's modification of the Gram stain (Hucker & Conn, 1923) for bacteria and lactophenol cotton blue (Booth, 1971) for fungi. If the cultures were considered to be pure, they were inoculated onto slopes, otherwise they were successively streaked and restreaked until pure cultures were obtained.

Stock cultures were maintained in two sets. One set, the working bench cultures, were inoculated onto respective agar slopes (PDA for fungi, PCA for bacteria and MRS for presumptive lactobacilli), incubated at 25° C and stored at 4° C. These were subcultured onto fresh media at 6 week intervals for bacteria, and 3 monthly in the case of fungi. The second set of isolates (fungi) were grown and stored as previously described, but subcultured yearly. The second set of bacteria were cultured in broth of the previously mentioned media until turbid. Subsequently, 1 ml of the turbid suspension of each isolate was added to 0.2 ml sterile glycerol contained in 2 ml capped vials (Treff Laboratory). The vials were then stored in a freezer at -20° C.

Characterization of Isolates

The aerobic heterotrophic bacteria were examined by the principles of numerical taxonomy. Lactobacilli, yeasts and filamentous fungi were identified as far as possible from diagnostic features.

Characterization of aerobic heterotrophic bacteria

Collection of data:

In addition to the 400 aerobic heterotrophic isolates, 41 named reference strains (Table 1) were examined for 111 unit characters, which have been described previously by Goodfellow et al. (1976) and Austin et al. (1978). All test media were inoculated from 24-48 h cultures grown at 25° C on PCA or PCB (plate count broth, i.e. PCA minus agar). The inoculated media were incubated at 25° C for 7 - 14 days, unless stated otherwise. The tests were done once, and repeated only if the results obtained were inconclusive. Tests which involved the clearing of insoluble or soluble compounds were carried out in Petri dishes. Growth in the presence of different concentrations of sodium chloride, on sole carbon sources, at different temperatures and degradation of Tween solutions were carried out in divided Petri dishes (Repli-dishes). Tests involving liquid media and/or incorporation of indicators were done in Repli-dishes (Sterilin) or in test tubes. PCA and PCB were used as the basal media for the tests whereever appropriate.

The tests included examination of colonial and micromorphology; motility; the ability to grow anaerobically; growth at 4°, 30°, 37° and 50° C, in 0, 2.5, 5.0, 7.5 and 10.0% (w/v) sodium chloride, at pH 4, 5, 6, 8, 9 and 10, on cysteine lactose electrolyte deficient agar (CLED, Oxoid) and MacConkey agar (Oxoid); production of arginine dihydrolase,

TABLE 1. Reference strains

Taxon	Strain designation/ source	Isolated from
Acinetobacter calcoaceticus	NCIB 5178	
*Aeromonas caviae	ATCC 15468	guinea pig
hydrophila	NCMB 89	diseased frog
*Arthrobacter crystallopoietes	ATCC 15481	soil
*globiformis	NCIB 8907	soil
*nicotianae	NCIB 9458	tobacco
*Bacillus alvei	CCM 2051	foul brood of bees
*amyloliquefaciens	ATCC 23350	soil
*brevis	CCM 2050	
cereus	CCM 2010	
*circulans	CCM 2048	
licheniformis	ATCC 9945	flour
*megaterium	CCM 2007	
polymyxa	ATCC 12321	spoiled starch
*pumilus	ATCC 7061	
sphaericus	ATCC 10208	
Brevibacterium flavum	ATCC 13826	
linens	ATCC 8377	cheese
Erwinia herbicola	NCPPB 656	Malus sylvestris
Escherichia coli	NCTC 8198	
*Flavobacterium aquatile	NCIB 8694	deep well, Kent
*breve	NCTC 11099	bronchial secretion
esteroaro-maticum	ATCC 8091	

Table 1(cont.)

Taxon	Strain designation/ source	Isolated from
Lactobacillus casei	CCM 1753	
fermentum	NCIB 6991	
Listeria denitrificans	ATCC 14870	
Microbacterium lacticum	NCIB 8450	
*Micrococcus luteus	ATCC 4698	
Mycobacterium sp.	Körmendy	fish
smegmatis	Körmendy	fish
Nocardia asteroides	ATCC 14759	pus from empyema
corallina	ATCC 4273	
*Planococcus citreus	NCIB 1493	seawater
*Proteus vulgaris	NCTC 4175	
Pseudomonas fluorescens	NCMB 1283	fish
putida	Stewart, 58	
Serratia marcescens	NCTC 1377	
Staphylococcus epidermidis	NCIB 2699	
Xanthomonas campestris	NCPPB 528	Brassica oleracea var. gemmifera
phaseoli	NCPPB 381	
phaseoli var. fuscans	NCPPB 382	

*denotes type strain

Sources: ATCC, American Type Culture Collection, Rockville, Md., USA; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; NCIB, National Collection of Industrial Bacteria, Aberdeen; NCMB, National Collection of Marine Bacteria, Aberdeen; NCPPB, National

Collection of Plant Pathogenic Bacteria, Harpenden; NCTC, National Collection of Type Cultures, Colindale, London; Dr. B. Körmendy, Central Veterinary Institute, Hungary; Dr. D.J. Stewart, Heriot-Watt University, Edinburgh.

catalase; fluorescein, ß-galactosidase, H_2S, indole, levan, lysine and ornithine decarboxylases, oxidase, phenylalanine deaminase and pyocyanin; fermentative and oxidative metabolism of glucose; degradation of aesculin, blood, casein, chitin, DNA, elastin, gelatin, lecithin, starch, Tween 20, 40, 60 and 80, tyrosine and urea; gluconate oxidation; methyl red test; nitrate reduction; Voges-Proskauer reaction; sensitivity to chloramphenicol (10 µg), chlortetracycline (10 µg), penicillin G (1.5 IU), neomycin (10 µg), furazolidone (2.5 µg), oxytetracycline (10 µg), streptomycin (10 µg) and sulfafurazole (10 µg); utilization as the sole source of carbon for energy and growth of calcium lactate, ethanol, D-fructose, D-galactose, D-glucose, glycerol, lactose, L-leucine, L-lysine, maltose, sodium acetate, sodium glutamate, sodium malonate, sodium tartrate, sucrose and D-xylose. Specific details of the tests are given below.

Colonial morphology and micromorphology:

Colonial morphology was determined from PCA plates after 14 days incubation at 25° C. The characters recorded included presence of white/off-white/cream, yellow, orange and pink-red pigments, shiny, mucoid, matt, round, raised, entire, spreading, wrinkled colonies; and production of diffusible pigment. Pyocyanin and fluorescein production

were observed under ultraviolet, on Pseudomonas P and F agar (Difco), respectively, using the modified method described by Gerhardt et al. (1981). Motility and micromorphology were determined microscopically. Wet mounts from 24 h old cultures in PCB were observed under the high power magnification (x40) lens of a light microscope for the presence of motile cells. Hucker's modified method (Hucker & Conn, 1923) of the Gram stain was used for distinguishing micromorphology, ie. such as cell size and intracellular inclusions. Acid fastness was tested using Ziehl-Nielsen's method as described by Gerhardt et al. (1981).

Biochemical tests:

Oxidation or fermentation of glucose was determined using the O/F basal medium developed by Hugh & Leifson (1953). Filter-sterilized (Millipore Millex 0.22 um porosity filter) glucose was added to the sterile basal medium to give a concentration of 1% (w/v) glucose. Duplicate test tubes were stab-inoculated, and the tube used for detecting fermentative utilization of glucose was over-layered with 1 cm of sterile mineral oil. Acid production, indicated by a yellow coloration of the medium in both tubes or in the covered tube, was indicative of fermentative metabolism. A yellow coloration in only the open tube indicated an oxidative metabolism. Tubes showing a blue or green coloration indicated no action on the carbohydrate.

Overnight cultures were tested for catalase production using a modified method described by Cowan (1974). Here, a drop of 3% (v/v) hydrogen peroxide was added to an isolated colony, and the production of effervescence within 5 min indicated a positive reaction.

Beta-galactosidase activity was detected using Lowes (1962) o-nitrophenol-β-D-galactopyranoside (ONPG) test. Thus, peptone water

containing ONPG was inoculated with the test organisms and incubated overnight. The change of the colourless substrate to yellow o-nitrophenol was indicated of a positive reaction, ie.:

$$\text{ONPG} + H_2O \xrightarrow{\beta\text{-galactosidase}} \text{galactose} + \text{o-nitrophenol}$$

For detecting gluconate oxidation, the medium described by Shaw & Clarke (1955) was used. Briefly, 1.0 ml of Benedict's quantitative solution was added to 5 ml of a 7 day old culture, mixed well, and boiled for 10 min. The formation of a brown, orange or tan precipitate constituted a positive reaction.

Hydrogen sulphide production was assessed by the blackening of a Kligler Iron Agar (Oxoid) slant after incubation for 7 days.

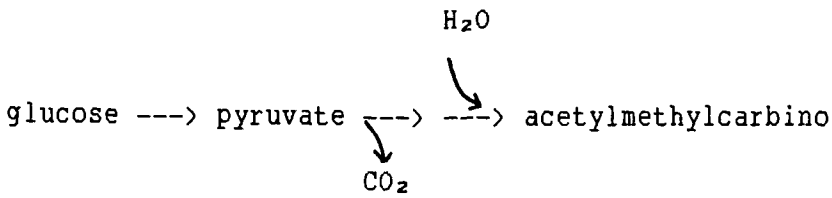
Nitrate reduction was examined using nitrate broth consisting of potassium nitrate, 0.1% (w/v); yeast extract, 0.1% (w/v); D-glucose, 0.5% (w/v). Absence of nitrate in the medium was tested using the test reagents given by Cowan (1974). After incubation for 7 days, a positive reaction was indicated by a red coloration formed after the addition of the test reagents. Where a negative result was obtained, approximately 5 mg l^{-1} of zinc dust was added to the previously tested medium, and a pink or red coloration developing within a few minutes indicated that nitrate was still present in the medium (i.e. not reduced by the organism). Absence of a red coloration showed that nitrate was absent in the medium (i.e. reduced by the organism beyond nitrite).

Indole production was determined using tryptone water 1% (w/v) (Gerhardt et al., 1981). After incubation for 5 days, the presence of indole was detected by the addition of 0.5 ml of Kovac's reagent (Gerhardt et al., 1981). After shaking the tube gently a red

coloration indicated a positive response.

Methyl red (MR) test medium (glucose-phosphate medium; Cowan, 1974) was used to detect the methyl red reaction. After 5 days incubation in the medium, 2 drops of methyl red solution were added, shaken and examined for a red coloration which indicated that glucose has been fermented to lactic acid

To test for the Voges-Proskauer reaction, glucose peptone broth (Abdel Malek & Gibson (1948)) consisting of glucose 10% (w/v) and peptone 1% (w/v) was used for growing the organism. After 5 days incubation at 25° C Barritt's method (1936) was used to detect the production of acetoin (acetylmethylcarbinol). Here, 0.6 ml of 5% (w/v) α-naphthol solution and 0.2 ml 40% (w/v) KOH aqueous solution were added and mixed. A positive reaction was indicated after 15 - 60 min by a strong red colour. This showed that the pyruvate had been decarboxylated and condensed to form acetylmethylcarbinol, i.e.:



The oxidase reaction (Kovacs, 1956) was tested with 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride (Sigma). A piece of Whatman No. 3 filter paper was impregnated with 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride and placed in a Petri dish. Inoculum was taken with a sterile platinum loop from an overnight culture on PCA and smeared across the surface of the impregnated paper. A positive reaction was recorded by the development of a dark purple colour within 30 seconds.

The deamination of phenylalanine was investigated on phenylalanine

agar slopes (Ewing et al., 1957) which were inoculated heavily with the test organisms and incubated overnight. Then, 0.2 ml of 10% (w/v) $FeCl_3$ aqueous solution was run over the culture. A positive result was indicated by a green colour on the slope and in the free liquid at the base. This indicated that the phenylalanine had been converted by oxidative deamination to phenylpyruvic acid, which formed a green colour in the presence of ferric chloride.

Levan production was tested using PCA basal medium supplemented with 5% (w/v) sucrose. After incubation for 7 days, the production of heavy (mucoid appearance) slime around the colonies or in the medium constituted a positive reaction.

Decarboxylation of L-lysine and L-ornithine was examined using Falkow's medium (modified from Falkow, 1958) which consisted of peptone (5 g), yeast extract (3 g), glucose (1 g), distilled water (1000 ml), ornithine or lysine (0.5% (w/v) each). The pH was adjusted to 6.7. After incubation for 7 days, a violet coloration indicated a positive response.

The production of arginine dihydrolase was tested in arginine broth (Niven et al., 1942), using method I described by Cowan (1974). After incubation for 24 h, 0.25 ml Nessler's reagent (BDH) was added and the development of a brown colour indicated a positive reaction.

Degradation tests:

Chitin (20 g) (BDH) was ground in 250 ml of distilled water for 3 days in a ball mill (Pascall Engineering Co. Ltd.). The paste was incorporated into melted water agar giving a final concentration of 1% (w/v). This was then poured as a thin layer on top of a base of PCA agar in Petri dishes. Inoculated plates were incubated at 25° C for 28

days, when a positive response was indicated by the disappearance of insoluble chitin around the area of growth on the medium.

Starch degradation was determined using PCA amended with 1% (w/v) soluble starch (Oxoid) and autoclaved at 115° C for 5 min. The plates were streaked and incubated for 7 days. After incubation, the plates were flooded with iodine solution to detect clearance of starch (Gerhardt et al., 1981).

The degradation of DNA was tested using DNase medium (Oxoid) prepared by adding 0.2% (w/v) DNA to PCA base prior to autoclaving. Inoculated plates were incubated at 25° C for 7 days. Then, the plates were flooded with 1M HCl and examined for a clear zone around the growth.

Aesculin degradation was determined using the method described by Gerhardt et al. (1981). Inoculated cultures were examined daily for 7 days for the presence of darkening around the bacterial growth. This brown coloration was due to the reaction of the aglycone (6:7-dihydroxy-coumarin) with iron (ferric citrate).

The degradation of tyrosine was detected using a modified method of Gordon & Smith (1955). PCA supplemented with 0.5% (w/v) L-tyrosine and sterilized at 115° C for 20 min was inoculated and after incubation for 28 days the plates were examined for the disappearance of insoluble tyrosine, which was indicative of a positive result.

The hydrolysis of Tween 20, 40, 60 and 80 was investigated using the medium and method described by Sierra (1957). After incubation for 14 days, the plates were examined, and a positive response indicated by opaque areas around the growth, which was due to the deposition of insoluble calcium salts.

PCA supplemented with fresh sterile skimmed milk (5% (w v); Oxoid)

was used to test for casein degradation. Here, 500 ml of double strength PCA was autoclaved (121° C/15 min) and cooled to 50° C before adding an equal volume of the skimmed milk. After incubation for 7 days, the inoculated plates were examined for zones of clearing around the growth.

The degradation of elastin was determined using PCA supplemented with 0.3% (w/v) elastin. Sterilization was at 115° C for 10 min. After 21 days of incubation, a positive reaction was indicated by the clearing of the insoluble elastin from around the growth.

The production of urease was tested using the medium described by Christensen (1946). Glucose and phenol red were added to urease basal medium. This was steamed for 1 h and cooled to 50° C before adding filter-sterilized (Millipore Millex 0.22 µm porosity filters) urea (BDH) to 1% (v/v). After 5 - 7 days incubation on slopes, a positive reaction was indicated by a red-violet colour.

Haemolysis of blood was examined on blood agar base (Oxoid) supplemented with 5% (v/v) sheep blood (Oxoid). The inoculated plates were examined daily for up to 7 days and β-haemolysis was indicated by a zone of clearing around the growth.

The degradation of gelatin was examined according to the method described by Collins & Lyne (1984). PCA medium supplemented with 5% (w/v) gelatin (Oxoid) was streaked, incubated for 7 days, flooded with saturated ammonium sulphate solution and examined for clearing around colonies.

Lecithinase production was tested using the medium described by Holderman et al. (1977) and the method given by Gerhardt et al. (1981). The egg yolk agar consisted of peptone (2% w/v), Na_2HPO_4 (0.25% w/v), NaCl (0.1% w/v)), $MgSO_4$ (0.5% w/v) solution, 0.10 ml), glucose (0.1%

w/v), agar (1.25% w/v). The egg surface was sterilized with ethanol and air-dried. The egg was carefully cracked, and the yolk separated, homogenized and added aseptically to the melted cooled agar. After incubation for 7 days, a positive response was indicated by cloudiness around the colonies.

Tolerance tests:

The sensitivity of bacterial isolates to a range of sodium chloride concentrations (i.e. 0, 2.5, 5.0, 7.5 and 10.0% (w/v)), was determined using suitably modified PCA. The presence or absence of growth was recorded after incubation for 7 days.

PCA was used for testing the ability of organisms to grow at 4, 30, 37 and 50° C. The presence or absence of growth was recorded after incubation for 28, 7, 7, 4 and 2 days, respectively.

The ability of the organisms to grow on MacConkey agar and CLED was recorded after incubation for 7 days.

Sensitivity of the isolates to antibiotics was carried out as follows. Aliquots (0.1 ml) of 24 h broth cultures were inoculated onto PCA plates and spread using a glass spreader. Multodisks (Oxoid) were placed onto the agar surface. These were incubated at 25° C for 24 - 48 h. Zones of clearing around the Multodisks of >3 mm were regarded as positive, i.e. the organisms were susceptible. The following antibiotics were tested: chloramphenicol (10 µg), chlortetracycline (10 µg), furazolidone (2.5 µg), neomycin (10 µg), oxytetracycline (10 µg), penicillin G (1.5 IU), streptomycin (10 µg) and sulfafurazole (10 µg).

Anaerobiosis was recorded after incubation for 7 days in an anaerobic jar containing an atmosphere of CO_2 and nitrogen.

Nutritional studies:

The ability of isolates to utilize sodium citrate, sodium acetate, sodium malonate, sodium glutamate, sodium tartrate, calcium lactate, L-leucine and L-lysine, as the sole carbon source for growth and energy was tested using the method described by Gordon & Mihm (1957). The basal medium consisted of NaCl, 0.1% (w/v); $MgSO_4.7H_2O$, 0.2% (w/v); $(NH_4)_2HPO_4$, 0.1% (w/v); KH_2PO_4, 0.5% (w/v); agar, 2.0% (w/v); distilled water; phenol red 0.0008% (w/v). The ingredients were dissolved and the pH adjusted to 6.8 before addition of the indicator. Sterilization was at 115° C for 20 min.

The utilization and production of acid from lactose, D-glucose, maltose, D-galactose, D-fructose, sucrose, glycerol, ethanol and D-xylose were investigated using method I of Cowan (1974). Peptone water containing 0.2% (w/v) bromo-cresol purple and PCB were amended with the carbon compounds (0.2% (w/v). The carbon compounds were sterilized by filtration through Millipore Millex 0.22 µm porosity filters, or if insoluble, autoclaved at 105° C for 25 min before addition to the basal medium. The media were poured into Repli-dishes. Inoculation was achieved using a multi-point inoculator (Denley). The inocula were prepared by suspending test organisms in sterile (121° C/15 min) Ringer's solution (Oxoid). After incubation at 25° C for between 7 and 30 days, a positive response was indicated by alkali production, hence a colour change.

Test Error

To assess for possible test error, 25 strains were ramdomly picked and examined in duplicate with the other bacteria. The probability (p) of an erroneous result was calculated using the procedure of Sneath &

Johnson (1972) and Sneath & Collins (1974).

Coding of Data

Most of the characters existed in one of two mutually exclusive states, and were coded as '1' and '0' for positive and negative, respectively. The remaining tests, eg. pigmentation, were divided into several mutually exclusive states, and isolates were coded as '1' for the particular character state exhibited, and '0' for the others. Five of the 111 unit characters were deleted from the data matrix because all strains gave identical results. The final $\underline{n}$ x $\underline{t}$ matrix contained 106 characters and 441 strains.

Computer Analyses

The data were entered onto cards, and then into the computer. The data were examined by the simple matching coefficient (S_{SM}; Sokal & Michener, 1958) which includes both positive and negative matches,

$$S_{SM} = \frac{a + d}{a + b + c + d} ,$$

and the Jaccard coefficient (S_J; Sneath, 1957) which excludes negative matches,

$$S_J = \frac{a}{a + b + c} ,$$

and by unweighted average linkage culstering (UPGMA; Sneath & Sokal, 1973). These algorithms were contained on the CLUSTAN 1C Programme Package (Wishart, 1978) available on the Heriot-Watt University Burrough's B3890 computer.

Lactobacilli

Cultures of presumptive lactobacilli recovered from MRS medium were examined for the production of ammonia from arginine, H_2S, indole and levan, growth at 15° and 45° C, degradation of aesculin, casein, gelatin and starch, and utilization as the sole source of carbon for energy and growth of amygdalin, L-arabinose, cellobiose, ethanol, D-fructose, D-galactose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose and D-xylose; and fermentation of glucose and gluconic acid. As far as possible modified MRS broth or agar (Oxoid) were used as basal media. Apart from those tests that were not included previously, the methods are described below.

Fermentation of gluconic acid and glucose was examined in PCB basal medium amended with the sugars (0.2% w/v) and phenol red (0.2% w/v). This medium was dispensed into test tubes with inverted Durham tubes. These were used to assess CO_2 production, which was indicative of a positive reaction. Production of NH_3 from arginine was investigated using the method described by Gerhardt et al. (1981). The cultures were inoculated into arginine broth (Evans & Niven, 1950) and incubated at 30° C for 3 days. Then a few drops of Nessler's solution (BDH) were added. A positive reaction was indicated by a yellow or orange coloration.

Characterization of Filamentous Fungi

Cultural and micromorphological characteristics of the moulds were assessed by examination under a stereomicroscope (7 - 45 magnification) of 10 day cultures on PDA supplemented with penicillin G and streptomycin sulphate. Subsequently, slide preparations, made with

specimens taken from these cultures, were stained with lactophenol cotton blue and examined at x400 magnification under a light microscope. This permitted the recognition of sporing and mycelial characteristics. In cases where it was difficult to prepare intact specimens of moulds, slide cultures were made. This involved culturing the organism on a small piece of agar (10 x 10 x 1-2 mm) which was placed on a sterilized slide in a sterile Petri dish. After inoculation, sterile cover slips were placed over the blocks. Following incubation, the preparation was stained with lactophenol cotton blue. Examination was under a light microscope (x400 magnification). Cultures which did not readily sporulate (notably Fusarium), were exposed continuously to near-ultraviolet light (Nirenberg, 1981).

Characterization of Yeasts

The 50 pure strains, obtained from dilution plates, were initially subcultured as short (~1 cm) streaks on Wallerstein Laboratories Nutrient agar (WLN; Oxoid). This procedure has been described by Campbell (1972). After incubation at 25° C for 3 days, the isolates were sorted into identical groups on the basis of their colonial morphology. The resulting groups were examined for 56 characteristics for the purpose of identification. Colony morphology was assessed from cultures on malt extract agar (Oxoid) and malt extract broth (Oxoid) grown for 7 days and 2 - 3 days, respectively. Slide preparations from these cultures were used to study micromorphology. Capsular material was observed using smears stained with Indian ink. The presence of pseudo- and true mycelium was determined from cultures on cornmeal agar (Oxoid). After incubation at 25° C for 10 days the streaks were

examined microscopically. The ability to ferment galactose, glucose, maltose, melibiose, raffinose and sucrose, and also to assimilate hexose (galactose), disaccharides (cellobiose, lactose, maltose, melibiose, sucrose, and trehalose), trisaccharides (melezitose and raffinose), polysaccharide (soluble starch), pentoses (L-arabinose, D-ribose, L-rhamnose and D-xylose), alcohols (ethanol and glycerols), organic acids (citric acid, lactic acid and succinic acid), and glycosides (arbutin and α-methyl-D-glucoside) followed the methods recommended by van der Walt & Yarrow (1984). The basal medium of Wickerham (1951) was prepared by combining mycological peptone (0.1% w/v) and yeast extract (0.5% w/v; Oxoid) containing 2 ml bromothymol blue (0.4% w/v) in 50 ml volume of distilled water; adjusting the pH to 6.8 and autoclaving at 121° C for 15 min. The volume was adjusted to 100 ml with 3% (w/v) filter-sterilized (Millipore Millex 0.22 µm porosity filters) sugars. Five ml aliquots each of the resulting mixture were aseptically dispensed into sterile test tubes with inverted Durham's tubes. These tubes were inoculated with heavy cultures (48 h), and incubated at 25° C for 3 - 7 days. Accumulation of gas in the inserted tube and a change in colour to green-yellow were recorded as positive fermentation reactions.

Replica-plating technique with the use of a multiple inoculation device (Denley Multipoint Inoculator) on an agar medium was employed for the assimilation test. The basal medium consisted of α-histidine (0.001% v/v), $(NH_4)_2SO_4$ (0.5% w/v), DL-methionine (0.002% v/v), DL-tryptophan (0.002% w/v), yeast salts (0.1% w/v), bromocresol purple (0.0001% w/v) and agar No. 1 (1.2% w/v). Carbon sources (sterilized by filtration or by Tindallisation) and 1.0 ml of vitamin solution were added to the sterilized basal medium before dispensing into divided

Petri dishes. Carbon free and glucose-incorporated media served as controls. The inoculated plates were incubated at 25° C for up to 14 days. Growth and a change in colour from purple to yellow were recorded as positive results.

In the case of arbutin utilization, 2 - 3 drops of 1% w/v ferric ammonium citrate solution were added to the basal medium immediately after sterilization. Detection of arbutin hydrolysis depends upon the fact that the aglucone moiety, hydroxyquinone gives a brown colour with the ferric salt.

The procedure for the assimilation of nitrogen compounds, i.e. L-lysine and potassium nitrate was similar to that described for carbon utilization. However, the basal medium in this case consisted of glucose (10 g), yeast salts (1 ml of stock solution), agar No. 1 (12 g), and 0.5 ml vitamin solution per litre of distilled water. The pH was adjusted to 6.0. Filter-sterilized (Millipore Millex 0.22 µm porosity filters) nitrogen source (0.05% w/v) was added and distributed after mixing into divided Petri dishes. Nitrogen free controls were also prepared. Plates were incubated for 7 days. Growth was recorded as a positive reaction.

The requirement of vitamins for growth was assessed using the nitrogen basal medium. The vitamin solution (1.0 ml l^{-1}) was added to the sterilized basal medium before pouring plates. A control plate, which contained no vitamin, was also prepared. The plates were inoculated and incubated at 25° C for 3 - 7 days. Growth on the test plates, but not on the controls was evidence of a requirement for vitamins. The composition of the vitamin solution comprised biotin, 20 µg, calcium pantothenate , 2000 µg, folic acid, 2 µg, m-inositol, 10,000 µg, niacin, 400 µg, p-aminobenzoic acid, 200 µg, pyridoxine

hydrochloride, 400 µg, riboflavin, 200 µg, thiamine hydrochloride, 400 µg l^{-1}.

Tolerance of the isolates to 20% (w/v) glucose and to 100 mg l^{-1} of cycloheximide was determined after incubation at 25° C for 7 days in appropriately modified Sabouraud broth (Oxoid) and on actidione agar (Oxoid), respectively. Evidence of growth was regarded as a positive reaction.

Growth at 30° and 37° C used the method of Kreger van Rij (1964). Malt extract agar (Oxoid) was inoculated with the test organisms and incubated at the respective temperatures for 4 days. Doubtful growth was subcultured and reincubated for confirmation.

Spore formation was examined following the method described by van der Walt & Yarrow (1984). Malt extract agar was used as the pre-sporulation medium on which the organisms were grown for 48 h. These actively growing cultures were then subcultured on sporulation agar (sodium acetate agar; Fowell, 1952) prepared as slopes. Slide preparations were made after incubation at 25° C for 21 days. These preparations were examined microscopically at x400 magnification. Cultures showing negative results were incubated for up to 1 month and then stained by the procedure of Schaeffer & Fulton (1933). Thus, heat fixed preparations were flooded with 5% (w/v) aqueous malachite green, heated to steaming for 30 - 60 sec., washed under running water for 30 sec., and counterstained with safranine for 30 sec. The mature spores stained blue-green and the vegetative cells red.

Identification of the Micro-organisms

Groups (phena) of aerobic heterotrophic bacteria not containing reference cultures, were identified as far as possible using the

diagnostic schemes in Cowan (1974), Starr et al. (1981), Krieg & Holt (1984) and Sneath et al. (1986). Lactobacilli were identified according to the schemes of Sharpe (1979) and Kandler & Weiss (1986). The filamentous fungi were identified using the diagnostic keys of Onions et al. (1981), Domsch et al. (1980), Raper & Thom (1949), Raper & Fennell (1965), Booth (1971) and Samson et al. (1984). The yeasts were identified using the schemes of Barnett et al. (1983), and Kreger van Rij (1984).

PHYSICAL ANALYSES ON DRIED BARLEY AND SCREENED MALT

Moisture Content

Duplicate 5 g lots of each sample were accurately weighed in a tared metal dish (5 cm diam., 1 cm depth) which was sealed tightly with a fitted lid. The dishes were dried in an oven at 105° C for 4 h. With lids fitted, the dishes were cooled in a desiccator before re-weighing. The moisture content was calculated as percentages.

Germination Tests

Tests for germinative capacity (peroxide method), germinative energy (4 ml) and water sensitivity (8 ml) were carried out in duplicate according to the methods recommended by the IOB (1977).

INFLUENCE OF SELECTED MICRO-ORGANISMS ON MALTING BARLEY AND MALT ANALYSIS

Barley

Triumph barley (English 1985 crop, as used in commercial maltings) was used throughout. The barley corn was stored in plastic bags at 2 - 4° C until required.

Micro-organisms

Fusarium nivale (strain Fm 5) and Geotrichum candidum (strain Fm 7) isolated from barley or malt were used for micro-malting.

Preparation of Micro-organisms for Use in Micro-malting

The moulds were cultured with shaking (Gallenkamp Orbital Incubator, 200 osc min^{-1}) in 1 l quantities of malt extract broth (MEB, Oxoid) at 25° C until profuse growth was evident (6 - 10 days). The mycelium and spores were harvested, washed twice in distilled water and suspended in sterile distilled water to the appropriate volume for steeping. The concentrations of the inocula were determined by plating appropriate diluted samples of the suspensions onto PDA. Aliquots were also plated onto PCA in order to confirm the absence of bacterial contaminants.

Micro-malting

Barleys were malted in 500 g lots following the schedule illustrated in Fig. 2.

The barley was weighed into a 2 l cylindrical plastic beaker which had been previously sterilized with Chloros (1% v/v) and rinsed with

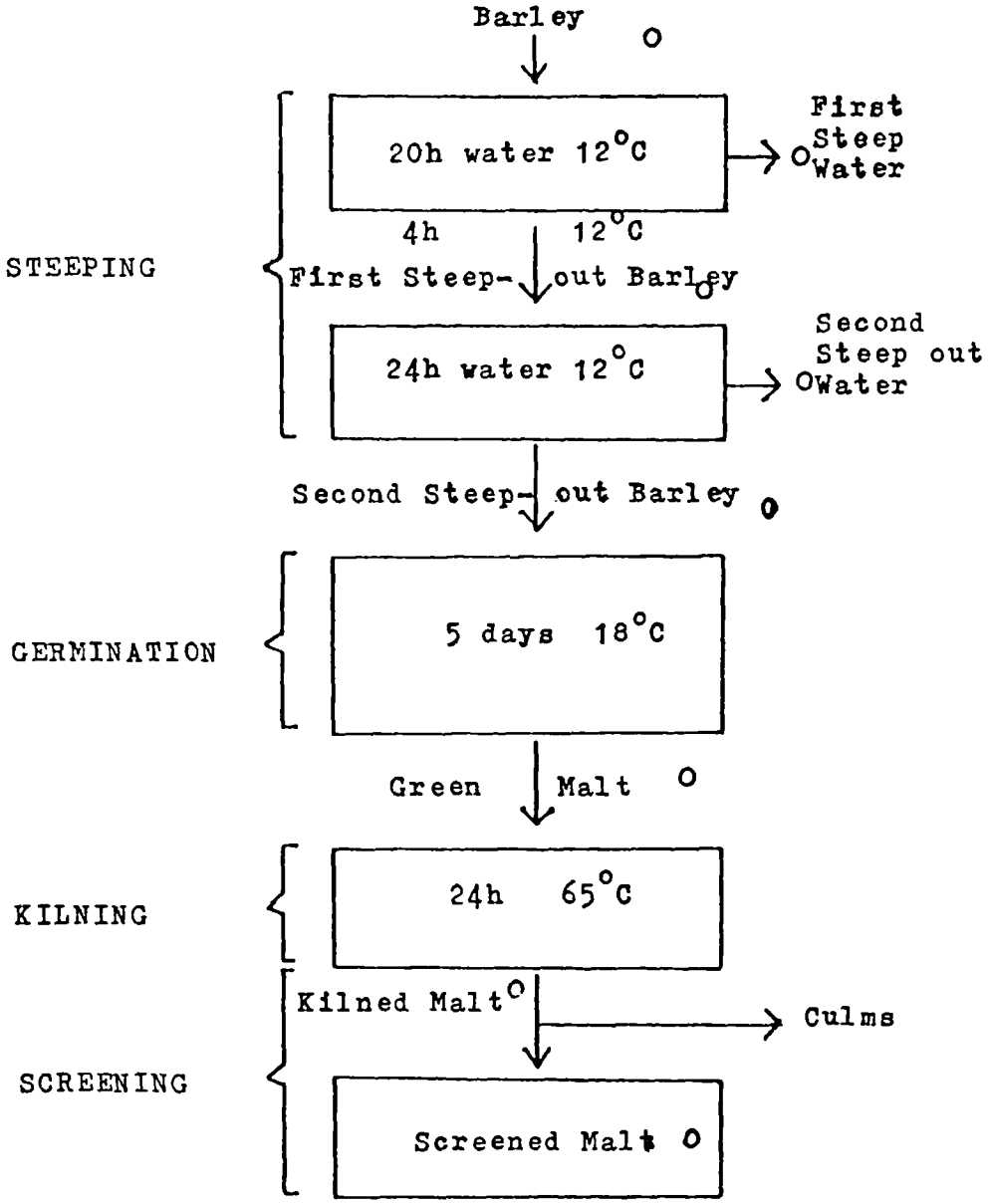


Figure 2. Diagram of Micro-malting Schedule.

sterile distilled water. An appropriate microbial suspension (2 ml g^{-1}) was added to the barley, mixed adequately before placing in an incubator at 12° C for 20 h. A control was also established in which the steep liquor did not contain any micro-organisms. After 4 h air-rest, a second steeping was carried out for 24 h duration at 12° C. At the end of this period, the steep liquor was drained off, and the corns transferred to germination boxes (36 by 21 by 5 cm in size; Astell Hearson) which were placed in a germination cabinet (Astell Hearson incubator) for 5 days at 18° C. Then the corns were transferred to an Astell kiln and the green malt kilned at 65° C for 24 h to produce a light malt. This kilned malt was screened to sieve out the culms, and the screened malts were stored in sealable plastic bags at 2 - 4° C.

Microbiological Analyses during Malting

Samples for microbiological analyses were collected at eight stages as indicated on the micro-malting schedule (Fig. 2). Approximately 40 g of the grain and 30 ml of water were collected at each sampling.

Direct and dilution plate techniques were used in determining the viable number of the micro-organisms. The media used included PDA, MSA, PCA and MRS for total fungi/yeasts, storage moulds, total bacteria and lactic acid bacteria, respectively. Direct plating was carried out only with the moulds. All plates were incubated at 25° C.

Malt Analyses

Total malt nitrogen and total soluble nitrogen of wort

The determinations were made using the Kjeldahl method on the

Tecator Kjeltec system, as described by the manufacturer.

Hot water extract

Coarse (0.7 mm) - fine (0.2 mm) extract analysis was carried out according to the IOB recommended methods of analysis (Wood, 1986) with modification in mashing equipment and specific gravity determination. A BRF mashing bath was used for mashing and specific gravity values were calculated from density readings of filtrates, obtained after injection into a Paar (DMA 46) Digital Density Meter. Fine-coarse difference was calculated to assess the extent of modification of the malt.

Cold water extract

Extracts were obtained according to the IOB recommended methods in the Brewing Room Book (Wood, 1986) with mashing equipment and specific gravity readings being the same as described above.

Alpha-amino nitrogen of wort

The International method described by the Analysis Committee of the European Brewery Convention, Analytica (EBC, 1975) was used to estimate the level of free α-amino nitrogen in the worts.

Malt colour

Wort was prepared as described for hot water extract and the colour determined according to the procedure detailed in the Journal of the Institute of Brewing (1977), using a Lovibond '2000' comparator.

Friability of malt

Friability of the malt was estimated using Friabilimeter (Apparatebau ING. F. Pfeuffer D-8711 Tiefenstockhelm). The method for the determination followed the manufacturer's instructions. The friability (P) in percent is given by the formula:

Friability (P) in percent = 100 - 2 x R,

and the evaluation table for friability is shown below:

81 - 100% = excellent

71 - 80% = good

65 - 70% = satisfactory

<65% = unsatisfactory

R = residual weight after milling 50 g sample.

Diastatic power

The assay for diastatic power (DP) was carried out according to the IOB recommended method (Wood, 1986) using Fehling's titration, to determine the level of reducing sugars.

One ml of the supernatant from malt infusion was digested against 2% (w/v) buffered starch solution (acetate buffer, pH 4.6) attemperated at 20 ± 0.25° C for 1 h. The reaction was stopped by the addition of 30 ml 0.1N sodium hydroxide solution and the volume subsequently increased with distilled water to 200 ml. The digested starch mixture was titrated against 5 ml of mixed Fehling's solutions with 3 drops of methylene blue (1% w/v) as an indicator. The end point was indicated by the decolorization of the methylene blue, and the reaction mixture just turning red. The choice of 1 ml malt infusion was settled at by trials such as to obtain the quantity of converted starch required to reduce 5 ml of Fehling's solutions to lie between 15 and 30 ml.

DP was expressed in IOB units calculated as:

$$DP\ (°L) = \frac{2000}{xy} - \frac{200}{xs}$$

where x = number of ml of malt extract

y = number of ml of converted starch to reduce 5 ml of Fehling's

s = titre for starch blank

Fermentability of boiled worts

The fermentability of the hot water extracts of malts was determined by fermenting the boiled worts with a standard yeast, 'M' yeast. The commercial malt previously collected from a malting factory was included as check malt.

The procedure for this determination followed the IOB recommended method of analysis (Wood, 1986) except that the pitching yeast was obtained by growing the yeast cells in malt extract broth for 5 days at 25° C using an orbital shaker at 200 rpm. The grown culture was centrifuged at 5000 rpm in an MSE 180 centrifuge for 20 min, and washed twice with sterile distilled water before taking the required weight.

The real attenuation was calculated using the formula:

$$\%\ \text{Fermentability (F)} = \frac{(OG - FG)x\ 81.9}{OG - 1.000}$$

and Fermentable Extract (FE) also calculated using the formula:

$$FE = E \times \frac{F}{100}\ \text{litre deg kg}^{-1}$$

where OG = Original Gravity

FG = Final Gravity

E = Extract "as is" = G x 10.13

G = Excess degrees of gravity of the filtrate taking water at 20° C as 1000 ie. G = 1000 (SG - 1).

Again, immersion of the wort in boiling water bath for 15 min was replaced by autoclaving at 115° C for 15 min, and filtering to remove the precipitated proteins before determining the original gravity. The wort was reautoclaved to sterilize before pitching with yeast.

Ethanol determination

The initial and final levels of ethanol in boiled wort, used for estimation of fermentability, were determined using Gas Chromatography (FlD-ECD, Perkin Elmer Fll). The gas carrier was nitrogen which delivered the hydrogen gas at the flame ionization detector at the rate of 20 ml per min. The glass column (3.66 m long, with internal diameter of 4 mm) was packed with 10% active compound (Carbo wax 1500) and inert compound (Chromosorb WHP, with 100 - 120 mesh) was used. A Mega Series integrator (Perkin Emer Fll) was used to analyse the data. One percent (v/v) of 96% (v/v) ethanol was used for the calibration. The mean of triplicate readings of ethanol levels was computed and the difference between the untreated and fermented worts ethanol levels calculated.

Physical Analyses

Moisture content during malting

Moisture content of dried barley and other samples taken during malting was determined as described previously and/or by the method described by Shands (1937) using 100 kernels.

Acrospire growth

Growth of acrospire was determined by measuring the length of the acrospire as a fraction of the length of the whole kernel, and the acrospire growth index calculated by the formula given by Shands et al. (1941) which is shown below:

Length of acrospire	Number of kernels	Factor	Index
Dead	x	0	x
0 - 1/4	x	0.25	x
1/4 - 1/2	x	0.50	x
1/2 - 3/4	x	0.75	x
3/4 - 1	x	1.00	x
overgrown	x	1.50	x
Total	x		x

Respiration and steep loss, root loss and recovery of malt

Respiration and steep loss, root loss during screening, and recovery of malt from barley were calculated using 100-corn weights and appropriate equations given by Shands et al. (1941) as:

$$\frac{\text{Wt. barley dry basis - wt. uncleaned malt dry basis}}{\text{Wt. barley dry basis}} \times 100 =$$

respiration and steep loss in percent

$$\frac{\text{Wt. uncleaned malt - wt. cleaned malt}}{\text{Wt. uncleaned malt}} \times 100 =$$

root loss in percent

$$\frac{\text{Wt. cleaned malt dry basis}}{\text{Wt. barley dry basis}} \times 100 = \text{recovery in percent}$$

DEGRADATION OF POLYSACCHARIDES BY MICRO-ORGANISMS ISOLATED FROM MALTING BARLEY

Screening for the Extracellular Enzymes in the Micro-organisms Isolated from the Commercial Malting Barley

Detection of xylanolytic activity in the isolates

The method described by Flannigan & Gilmour (1980) for simple plate test for xylanolytic activity in wood-rotting basidiomycetes was used. Each 9.0 cm plate was inoculated centrally with a 4 mm plug cut from the edge of a 4 - 7 day culture on PDA (Oxoid) in the case of filamentous fungi; a loopful of spore/cell suspension in 0.01% (v/v) Tween 80 (sporulating fungi/yeasts) and incubated at 25° C for 7 - 10 days. At the end of incubation the plates were flooded with 96% (v/v) ethanol to precipitate undegraded xylan, left overnight and observed for clear zones around the colony.

To confirm the results as positive, 4 mm plugs were cut from the margin of the original culture before flooding with ethanol and transferred to equal size holes cut in plates of the same medium ammended with 0.005M sodium azide l^{-1}. The plates were incubated at 25° C for 3 days before flooding with ethanol as before. Clearing zones were visible confirming a positive response.

The same basic principles used in testing fungi were applied to

bacteria, but suspensions were prepared from 2 - 3 day cultures of appropriate bacteria in 0.1% (w/v) Ringers solution, and streaks made on PCA with xylan as sole carbon source.

Detection of amylolytic activity in the isolates

This method was based on the reaction of starch with iodine to form starch-iodine complex which gave a blue-black colour in the medium, while the hydrolysed starch gave a clear or yellow zone around the colony, indicating an amylolytic activity.

Modified plate count agar (mPCA,) was prepared by substituting 0.1% (w/v) soluble starch (BDH) for the carbon source. These plates were inoculated with appropriate moulds, yeast, and bacteria. The medium for moulds and yeasts was adjusted to pH 6.0. Inoculation and incubation were done as previously described in the preceding section. After incubation the cultures were flooded with dilute Lugol's iodine solution. Yellow or clear zones around the colony were recorded as a positive response.

Detection of glucanase activity in the isolates

The modified method of Bol (unpublished) used for screening β-glucanase producing micro-organisms was used in this investigation. The method is similar to the procedure described by Farkas et al. (1985), Teather & Wood (1982) and Carder (1986). The test is based on the interaction of direct dye congo red with intact β-D-glucan to give congo red-glucan complex. The hydrolysed β-glucan forms no such complex and gives a clear zone.

Medium (Bol's)

The medium for bacteria comprised:

β-glucan	0.5 g
tryptone	8.5 g
K_2HPO_4	1.25 g
NaCl	2.5 g
Bacto soytone	1.5 g
agar No. 1	7.5 g
Dist. H_2O	500 ml
pH	6.8

and medium for fungi was composed of:

β-glucan	0.5 g
yeast extract	2.5 g
agar No. 1	9.0 g
Dist. H_2O	500 ml
pH	6.8

Both media were sterilized at 121° C for 15 min. Plates were inoculated and incubated as described earlier. After incubation at 25° C, the plates were flooded with congo red (0.2 mg ml^{-1}). After 15 min the congo red solution was poured off, and the plates further flooded with 1M NaCl, and examined after 15 min, although in some cases of doubtful results, they were left for up to 2 h (in such cases, N HCl was added to stop enzymatic reaction and the colour of congo red dye changed to blue-violet (pH 1.0) (Sazci et al., 1986). Clear zones around the colonies indicated β-glucanase production.

The barley-β-glucan used throughout this study was prepared in this laboratory by the modified method of Preece & Mackenzie (1952),

which is described in a subsequent section of the Materials and Methods.

Quantitative Determination of β-glucanase Activity in Some Selected Isolates

Isolates:

Microbacterium lacticum (A11)

Bacillus sp. (A6)

Bacillus sp. (W12)

Arthrobacter globiformis (A9)

Microbacterium imperiale (A10)

Fusarium nivale (Fm 5)

Geotrichum candidum (Fm 7)

Fusarium avenaceum (Fm 6)

Five bacteria and three filamentous fungi were isolated from the commercial malting barley. *Bacillus* sp. (W12) was supplied by Dr. F.G. Priest (Heriot-Watt University).

Determination of the Optimal Growth Period for the Microbial Production of β-glucanase at 25° C

Principle

The reduction or degradation of β-glucan to glucose molecules by β-glucanase was used as the criterion for measurement of growth. The amount of glucose released was determined using the Somogyi-Nelson colorimetric method for reducing sugar (Whistler & Wolfrom, 1962).

Procedure for growing the organism

The broth form of the Bol's medium was used in growing the organisms with pH adjusted to 6.6 for fungi and 7.0 for bacteria. Ten ml of the 0.1% (w/v) glucan broth was inoculated with 0.2 ml of 4.0×10^7 ml^{-1} washed bacteria previously grown in plate count broth (PCB, Oxoid). The fungal broth (10 ml) was inoculated with a 4 mm agar plug of mould previously grown on Agar No. 1 plates. Bacterial cultures were incubated in a water bath (orbital shaker, 100 rpm) attemperated at 25° C for up to 4 days. The same conditions were used for fungal incubation except that the speed of the shaker was 50 rpm and incubation lasted for up to 7 days.

Each day of the incubation period cultures were withdrawn from a series and filtrates prepared from these.

Preparation of enzyme solutions

After the required incubation period (see above), the bacterial culture was aportioned into approximately 5 ml aliquots. One portion was centrifuged (10,000 rpm directly for 15 min), while the other portion was further subdivided into aliquots of 2.5 ml. One drop of toluene solution was added to a portion while the other portion was toluenised only after inactivating the enzymes by placing in boiling water for 15 min. The toluenised mixtures were then centrifuged (10,000 rpm, 10° C). The supernatant served as the enzyme sources.

Substrate preparation

Barley ß-glucan (0.1 g) was dissolved in 50 ml of distilled water and sterilized at 121° C for 15 min. Filter-sterilized citrate

phosphate buffer (50 ml) at pH 6.6 was added to give a 0.1% (w/v) solution for fungi, and at pH 7.0 for bacteria.

Assay

The buffered barley-β-glucan was attemperated at 37° C in a water bath was dispensed at 0.5 ml aliquots into test-tubes containing 0.5 ml of appropriate culture filtrate. After mixing thoroughly, they were incubated at 37° C for 1 h in a water bath. Equal volumes of culture filtrate were boiled for 15 min to inactivate the enzymes and subsequently treated as the test mixture. This served as the control and reagents in buffer solution as the blank. At the end of the reaction, the tubes were placed in boiling water for 15 min to stop the enzymatic reaction. Following this, reducing sugars in 1 ml aliquots were estimated by the Somogyi-Nelson method. Absorbance was read at 600 nm. All tests were in duplicate. Glucanase activity was determined from a glucose standard curve in $\mu g\ ml^{-1}$ glucose released per assay.

Preparation of glucose calibration curve

A calibration graph was prepared using D-glucose in 0.05M citrate phosphate buffer or appropriate buffer used in the assay. One ml aliquots, in triplicate, of each of the solutions with concentrations ranging from 0 - 100 µg glucose ml^{-1} were put through the Somogyi-Nelson reducing sugar procedure together with a water blank.

Chromatographic examination of enzyme digests

The residue of the reaction mixture was de-ionised by the addition

of granules of mixed-bed resin and centrifuged (6,000 rpm) for 15 min. It was then spotted along with standard 0.1% (w/v) sugars (glucose, xylose, melibiose, fructose, galactose and arabinose), on Whatman No. 1 paper chromatograms. These were subsequently developed by descending chromatography for 18 h in ethyl acetate:pyridine:water (10:4:3 v/v). The reducing sugars were then detected by the silver nitrate dip method of Trevelyan et al. (1950).

Effect of pH on the Production of β-glucanases by Micro-organisms at 25° C

The methods of glucanase production and glucose concentration determination were analogous to those described under growth period estimation. However, the media and buffers were prepared at pH 5.0, 5.6, 6.0, 6.6 and 7.0.

Estimation of β-glucanase Activity Brought About by Microbial β-glucanases using Brookfield Viscometer at 37° C

Viscometric method

This method is based upon the principle of Bathgate (1979) that used malt endo-β-glucanase to achieve a reduction of the kinematic viscosity of barley β-glucan substrate.

Substrate

Barley β-glucan (0.5% w/v) dissolved in 0.05M citrate phosphate buffer, pH 7.0 (bacteria) and pH 6.6 (fungi), was prepared as described previously under substrate preparation.

Organisms and culture filtrate

The micro-organisms and the enzyme filtrates preparation were as previously described in this section.

Assay

The substrate solution (3 ml) was mixed with 0.15 ml enzyme preparation. Boiled enzyme filtrate mixed with the substrate was used as a blank. The reaction mixture (0.7 ml) was immediately injected into a Brookfield viscometer well attemperated at 37° C, after recording the reading for the blank sample. The reductions in viscosity were recorded at an interval of 10 min. Duplicate runs each lasted for about 2 h. The unit of β-glucanase activity (Clarke & Stone, 1962) was expressed as that amount of enzyme required to produce a 10% reduction in initial (blank) specific viscosity in 100 min. The reduction produced within the linear portion of the curve of change in specific viscosity against reaction time was used to calculate the percentage reduction in specific viscosity and hence the time required to reduce the specific viscosity of the sample by 10% (t). The formula 100/t (Clarke & Stone, 1962) was then used to calculate the unit of enzyme activity per assay.

Preparation of barley-β-glucan

Barley β-glucan was extracted from grains by a variation of the method reported by Preece & Mackenzie (1952). The grain, Triumph variety, 1984 harvest was used. The barley was finely ground in a Buhler-Miag universal laboratory Disc mill (Type DLFU). The milled barley flour was boiled under a reflux in 70% ethanol (~1 l kg^{-1}

barley) for 30 min. to inactivate the enzymes present in the flour and subsequently the ethanol was allowed to evaporate off and the flour cooled to 40° C. It was then extracted at 40° C (1 l per kg barley) for 30 min. Three extractions were made. The extracted mixture was centrifuged (2,500 g for 20 min) and the supernatant incubated with filter-sterilized hog pancreatic α-amylase (6 g per 500 ml) for 16 h under an atmosphere of toluene. Iodine test was performed to ascertain the presence/absence of starch in the extract. The extract was boiled to stop the reaction and then centrifuged to remove precipitated proteins and other debris.

Ammonium sulphate (30% w/v) was added at room temperature to precipitate β-glucan and the mixture was kept at 2° C to speed up precipitation which was harvested by centrifugation. The precipitate was redissolved in distilled water and dialysed against running tap water for 24 h. Addition of ammonium sulphate and dialysis step was repeated to ensure purity. Dialysis was repeated until addition of barium chloride failed to reveal the presence of sulphate. An equal volume of acetone was added after the final dialysis to remove undesirable coloration and precipitate β-glucan. The absence of contaminant pentosan was demonstrated by paper chromatography of an acid hydrolysate. The dialysed β-glucan was freeze-dried overnight. Approximately 25 g dried β-glucan was recovered per 6 kg barley.

Acid hydrolysis of polysaccharides

Soluble polysaccharides were completely hydrolysed in 1.8M sulphuric acid at 100° C for 3 h. In this case, 10 mg β-glucan were dissolved in 1.8M sulphuric acid (1 ml) and hydrolysed in boiling water bath for 3 h. The hydrolysates were neutralized with barium carbonate

and the insoluble barium sulphate that formed removed by centrifugation.

Effect of Temperature on the Growth and Enzymatic Activity of F. nivale and G. candidum on Different Media

PDA glucan agar (0.1% w/v) and starch agar (0.1% w/v) (both prepared as described previously) were the solid media used for growing the organisms. Triplicate plates were each centrally inoculated with a 4 mm agar plug from 5-day cultures previously grown on Agar No. 1 inoculated with a plug from a PDA plate culture (to give non-sporulating mycelium).These plates were then incubated at appropriate temperatures and the diameter of growth measured each day. Plates at 37, 50 and 60° C were incubated for 2 - 4 days, and those at 4, 10, 18, 20, 25 and 30° C for 7 days.

Detection of Enzymatic Activities of F. nivale and G. candidum at Various Temperature Levels on Different Media

At the end of incubation periods as indicated in the preceding section, the β-glucan plates were flooded with congo red stain for the detection of glucanase activity while starch plates were flooded with dilute iodine solution for the detection of amyloytic activity.

Effect of Temperature on β-glucanase and Amylase Activities Brought About by Culture Filtrate from F. nivale in Liquid Medium

Glucanase activity

Duplicate 0.5 ml aliquots of active enzyme filtrate and controls

were prepared, and assayed for reducing sugars as described previously, after incubation with substrate at 10, 18, 20, 25, 30, 37 and 50° C for 1 h.

Amylolytic activity

Reduction brought about by amylases on iodine staining power of soluble starch and the levels of reducing sugars measured by Somogyi-Nelson reducing sugar method, were determined.

Assay:

Substrate (0.5 ml buffered soluble starch) was mixed with 0.5 ml of enzyme filtrate pH 6.0 prepared as previously described. These were prepared in sets of four and incubated at the temperatures indicated above for 10 min together with reagent blanks and inactivated controls. At the end of incubation, 10 ml of iodine reagent (0.254 mg ml^{-1} iodine in 4.0 g l^{-1} potassium iodide) were added to two tubes of each set and absorbance of the iodine-starch complex was measured at 600 nm. The unit of amylase activity was expressed as the reduction in absorbance occurring over the course of the assay. The remaining two tubes were analysed for reducing sugar by Somogyi-Nelson reducing sugar procedure. A glucose standard curve was used to determine the activity of the enzyme as $\mu g\ ml^{-1}$ glucose released per assay.

Preparation of soluble starch substrate:

Buffered starch solution was prepared by creaming 100 mg soluble starch in 20 ml distilled water and adding to 30 ml boiling distilled water, stirring constantly. The mixture was made to boil for 2 min and cooled under cover to 20° C to avoid skin formation. Then 50 ml

citrate phosphate buffer, pH 6.0 (17.9 of 0.1M solution of citric acid + 32.1 ml of 0.2M solution of dibasic sodium phosphate + 10mM $CaCl_2$), was added to give a 0.1% starch solution.

Effect of Incubation on the β-glucanase Activity Using Culture Filtrate from F. nivale at 37° C

The enzyme filtrate and 0.1% glucan substrate were prepared as described previously. The substrate (500 ml) was added to 500 ml of enzyme filtrate in 2 l flasks. To prevent contamination 20 ml of toluene were added. A control flask was also prepared in which boiled enzyme filtrate was substituted for active enzyme filtrate. The two flasks were incubated under an orbital shaker (150 rpm) at 37° C for 16 h during which time 5 ml samples were withdrawn at intervals indicated in Appendix 13. The Somogyi-Nelson reducing sugar method was used to analyse the digest. The unit of β-glucanase activity was determined from the glucose standard curve as $\mu g\ ml^{-1}$ glucose released $0.5\ ml^{-1}$ extract.

Determination of α-amylase Activity

This assay is based upon a reduction in iodine staining brought about by the action of α-amylase on purified β-limit dextrin in the presence of excess β-amylase.

Preparation of β-limit dextrin

Following the method of Nimmo (1983) 100 g soluble starch was suspended in 250 ml of distilled water and added to 1.5 l of boiling distilled water. Boiling was continued with stirring for 2 min to ensure complete gelatinisation of the starch. After cooling, 100 ml

of1M sodium acetate buffer pH 5.3, was added to give a final buffer concentration of 0.05M. One hundred mg of β-amylase, which had been dissolved in 2 - 3 ml of distilled water was added. The mixture was incubated with 37 ml toluene at 37° C for 3 days and then dialysed against running tap water for 16 h. The incubation and dialysis steps were repeated until the digest showed no further increase in reducing power, as detected by the Somogyi-Nelson method. The solution was then boiled for 5 min, centrifuged (10,000 g, 20 min) and freeze-dried. The freeze-dried preparation was further purified by re-dissolving in distilled water followed by a final centrifugation (10,000 g, 20 min) and freeze drying. A yield of 30 - 40 g β-limit dextrin was obtained.

Alpha-amylase Assay

Reduction in iodine staining:

A mixture (IOB method; Wood, 1986) of β-limit dextrin (4 mg ml^{-1}) and β-amylase (2 mg ml^{-1}) in 35 ml assay buffer (10mM $CaCl_2$ in 20 mM citrate phosphate buffer pH 6.0) (35 ml) was added to 35 ml of enzyme filtrate in a 100 ml flask and mixed. This was incubated in an orbital shaking water bath (100 rpm) after adding 1 ml toluene. Reagent blanks and controls containing inactivated (boiled) enzyme filtrate were also incubated along with the control. Samples (1 ml) were withdrawn at 10 min intervals, in duplicate, for 3 h duration and analysed by adding 10 ml of iodine reagent (0.254 g l^{-1} iodine in 4.0 g l^{-1} potassium iodide) and absorbance read at 600 nm immediately. The unit of α-amylase activity was expressed as the reduction in absorbance occurring over the course of the assay.

Effect of Concentration of Culture Filtrates of F. nivale and G. candidum on Iodine Staining in β-limit Dextrin

The assay was carried out as described above except that the volumes of enzyme filtrate varied as follows: 0, 0.1, 0.2, 0.3, 0.4, 0.5 ml; and the substrate (β-limit dextrin) volume added varied accordingly to make up a final volume of 1 ml mixture in each case. In addition, all tubes were incubated at 37° C for 10 min.

Amylolytic Activity of F. nivale and G. candidum at 37° C, Measured as Reduction in Iodine Staining and Reducing Sugar Levels

a) Effect of length of incubation

Appropriate enzyme filtrate (35 ml) and 0.1% (w/v) buffered starch solution (35 ml), prepared as previously described, were mixed and incubated in a shaking water bath (100 rpm) with 1 ml toluene. Reagent blanks and inactivated controls were incubated along with the test mixtures. A sample (4 ml) was withdrawn at 10 min intervals over 2 h, then at 30 min interval over 1 h duration for iodine staining and reducing sugar analysis.

The relationship between reduction in iodine staining of soluble starch (absorbance) and reducing sugar (μg glucose released ml^{-1}) was determined by plotting a graph to link the two parameters.

b) Effect of volume of culture filtrate

Iodine staining values and reducing power were assayed in tubes containing 0.1, 0.2, 0.3, 0.4 and 0.5 ml culture filtrate and substrate to make up the total volume to 1.0 ml. The active mixtures and controls were incubated at 37° C for 10 min.

Degradation of Raffinose and Sucrose by F. nivale and G. candidum

The method described by Flannigan (1977) for assaying enzyme activity was basically followed.

Preparation of the enzyme filtrate

Plate count broth (Oxoid) void of carbon source, was amended with 0.1% (w/v) sucrose or raffinose (BDH). The medium (100 ml) was inoculated with ten 4 mm plug test organisms (5-day culture on Agar No. 3) and incubated at 25° C under a shaker with very low speed, 200 rpm) for 5 days. At the end of incubation the culture was centrifuged (6,000 rpm) and the supernatant was dialysed overnight against 0.05M acetate buffer pH 5.6 both containing toluene.

Assay:

The reaction mixture consisting of 15 ml enzyme filtrate, 15 ml 0.1% sucrose or raffinose, pH 5.6, in 0.05 acetate buffer and 0.6 ml of toluene was incubated at 37° C in a water bath for 6 h, along with controls containing enzyme inactivated in a boiling water bath (15 min). Duplicate 1.0 ml samples were withdrawn from the reaction mixture hourly and immersed in boiling water to stop the reaction (15 min). Subsequently, the reducing sugars were estimated by the Somogyi-Nelson colorimetric method. A standard calibration curve was prepared using fructose in 0.05M acetate buffer (pH 5.6). The unit of enzyme activity was expressed as $\mu g\ ml^{-1}$ fructose released per assay.

The digest at the end of incubation was spotted along with fructose, glucose, galatose, and melibiose standards on Whatman No. 1 paper and put through chromatography as described previously.

Determination of the Protein Contents of the Culture Filtrate from 5 d Cultures at 25° C by Lowry Method

Culture filtrates from Fusarium nivale and Geotrichum candidum were tested for protein contents, using the Lowry colorimetric method (Miller, 1959). One ml aliquots of alkaline copper reagent composed of 10 parts of 10% sodium carbonate in 0.5N sodium hydroxide and 1 part of 0.5% copper sulphate in 1% potassium tartrate were added to 1 ml aliquots of protein solution. After the mixture had stood for 10 min, 3 ml aliquots of a 1 to 11 dilution of Folin phenol reagent were added to the samples as forcibly as possible. The resulting mixtures were heated at 50° C for 10 min in a constant temperature water bath. The mixtures were subsequently cooled to room temperature, and absorbance read at 600 nm. Results were calculated from a standard curve prepared using bovine serum albumin (Sigma; Fraction V,0 - 100 $\mu g\ ml^{-1}$).

RESULTS

QUANTITATIVE STUDIES

Numbers of Micro-organisms Associated with Barley Malt Production

The numbers of micro-organisms associated with malting barley, as determined by dilution plating are included in Table 2. Generally, there was a fluctuation in numbers of the micro-organisms throughout the malting process. However, the populations of lactic acid bacteria and yeasts showed a gradual rise in numbers from the dried barley to green malt, after which a small drop was recorded with kilned malt. With the exception of filamentous fungi, the numbers of organisms on green malt were much higher than on the dried barley.

The initial populations of micro-organisms associated with dry barley were estimated as 1.8×10^6 aerobic heterotrophic bacteria $kernel^{-1}$, 2.0×10^2 filamentous fungi $kernel^{-1}$ and 4.7×10^3 yeasts $kernel^{-1}$. During the first steep, populations of aerobic heterotrophic bacteria declined slightly to 6.7×10^5 $kernel^{-1}$, whereas numbers of lactic acid bacteria, filamentous fungi and yeasts increased substantially to 4.2×10^3, 8.0×10^2 and 4.6×10^5 $kernel^{-1}$, respectively. Barley, after the second steep, indicated some further increase in the population sizes of all four groups of micro-organisms. Green malt scored another increase although the numbers of filamentous fungi declined slightly. After kilning with screening, the numbers declined except in the case of filamentous fungi, which increased substantially. Large numbers of micro-organisms were present in the steep-waters. Apart from the slight decline in numbers of lactic acid bacteria, the populations of aerobic heterotrophic bacteria,

TABLE 2

Numbers of micro-organisms associated with barley malt production. The data were collected by incubating inoculated media at 25°C for 14 d.

Sample	No. of aerobic heterotrophic bacteria/ kernel or ml	No. of lactobacilli/ kernel or ml	No. of filamentous fungi/ kernel or ml	No. of yeasts/ kernel or ml
Barley (dry)	1.8×10^6	2.0×10^2	2.0×10^2	4.7×10^3
Barley (first steep)	6.7×10^5	4.2×10^3	8.0×10^2	4.6×10^5
Barley (second steep)	6.6×10^6	7.8×10^4	1.7×10^3	1.1×10^6
Green malt (5-day)	5.7×10^7	8.7×10^6	1.5×10^2	3.9×10^6
Kilned malt	5.6×10^6	1.6×10^5	2.0×10^2	3.2×10^4
Screened malt	5.5×10^6	5.7×10^4	8.3×10^2	1.8×10^4
Bore-hole water	1.1×10^1	0	0	0
Water (first steep)	1.7×10^7	2.9×10^6	8.0×10^2	5.7×10^5
Water (second steep)	2.0×10^7	2.6×10^6	9.0×10^2	1.2×10^6

filamentous fungi, and yeasts increased slightly in the second steep-water compared with the first steep-water. However, the initial bore-hole water was virtually devoid of micro-organisms. These data were statistically significant at the 95% level of significance (Student's t-test, Appendix 1).

Effect of Incubation Temperature on the Numbers of Micro-organisms Associated with Barley Malt Production

The effect of incubation temperature on the enumeration of microbial populations is depicted in Table 3. The highest populations of aerobic heterotrophic bacteria, filamentous fungi and yeasts were consistently recovered at 25° C at the three levels of malting examined (dried barley, kilned malt and screened malt). With screened malt, however, the highest population of lactic acid bacteria was recorded after incubation at 37° C. At 60° C, mostly thermophilic actinomycetes were recovered from all sample types, with the population peaking in kilned malt. On the contrary, the number of psychrophilic bacteria declined steadily from dried barley until the kilning stage, with an increase again on the screened malt. The highest number of psychrophilic yeasts was obtained from screened malt. There were no detectable psychrophilic or thermophilic filamentous fungi in any of the samples. Whereas psychrophilic lactic acid bacteria were recovered from screened malt, no thermophilic types were detected in any of the samples.

Table 3

Effect of incubation temperature on the enumeration of microbial populations associated with barley malt production. The total viable counts have been expressed for one kernel.

Sample	Temperature of incubation (°C)	No. of aerobic heterotropnic bacteria	No. of presumptive lactobacilli	No. of filamentous fungi	No. of yeasts
Barley (dry)	4	3.5×10^{4}	-	-	1.9×10^{3}
	15	3.3×10^{4}	1.7×10^{2}	-	2.0×10^{3}
	25	1.8×10^{6}	2.0×10^{2}	2.0×10^{2}	2.0×10^{3}
	37	3.0×10^{5}	1.0×10^{2}	-	-
	50	-	-	-	-
	60	3.0×10^{2}	-	-	-
Kilned malt	4	1.2×10^{4}	-	-	-
	15	2.1×10^{5}	4.3×10^{4}	1.0×10^{2}	1.3×10^{3}
	25	5.6×10^{6}	1.6×10^{5}	2.0×10^{2}	3.2×10^{4}
	37	4.1×10^{5}	3.5×10^{4}	-	6.0×10^{2}
	50	2.8×10^{4}	-	-	-
	60	1.7×10^{4}	-	-	-
Screened malt	4	6.6×10^{4}	2.2×10^{3}	-	4.8×10^{3}
	15	2.6×10^{5}	5.5×10^{4}	5.0×10^{2}	5.5×10^{3}
	25	5.5×10^{6}	5.7×10^{4}	8.3×10^{2}	1.8×10^{4}
	37	1.0×10^{6}	1.2×10^{5}	-	2.3×10^{3}
	50	7.0×10^{2}	-	-	-
	60	3.0×10^{2}	-	-	-

-Not detected

Electron Microscopy

Scanning electron microscopy was carried out to reveal the possible micro-organisms situated on the surfaces of the barley kernels. Examination of the micrographs revealed the presence of only low numbers of micro-organisms on the surface of dried barley grains, but notably higher populations, particularly of bacteria and fungal hyphae, on the inner surface below the husk (Fig. 3a). There was no obvious spatial distribution, however, with the bacteria clustered as micro-colonies with up to 200 cells, randomly distributed across the entire surface. A dense coverage of organisms, i.e. bacteria and yeast/fungal spores appeared on steeped grains, particularly damaged areas (Fig. 3b), and green malt. After kilning, abundant branching fungal hyphae (Fig. 4) were observed below and on the surface of the husk. Many micro-colonies of bacteria (Fig. 5) occurred on the surface. However, below the husk, negligible numbers of bacteria were seen.

QUALITATIVE STUDIES

Examination of Data by Numerical Taxonomy

Test error:

Twenty-five isolates were examined in duplicate to assess the possible experimental error. The average probability (p) of an erroneous test result was 4% calculated from the pooled variance (S^2 = 0.037) of all the unit characters scored for the duplicate strains (Appendix 2). The 25 pairs of duplicate isolates showed a mean observed similarity of approximately 96% S_{SM}. Values of S_i^2 varied from zero for most of the tests, through 0.08 for motility, β-

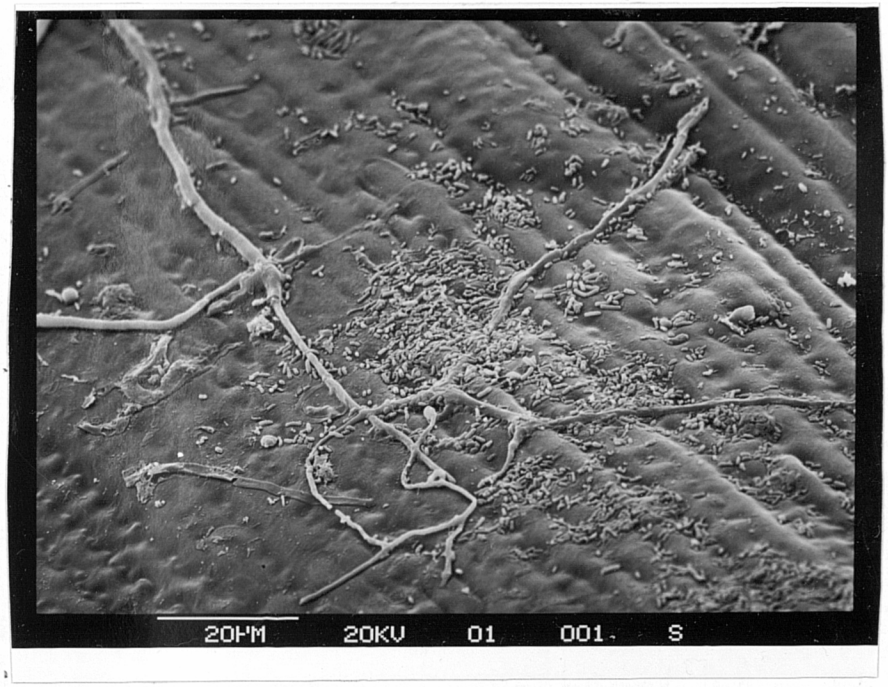


Figure 3a. Scanning electron micrograph of a dried barley grain with husk removed, showing the presence of numerous micro-organisms (notably bacteria) on the inner surface. Bar = 20um.

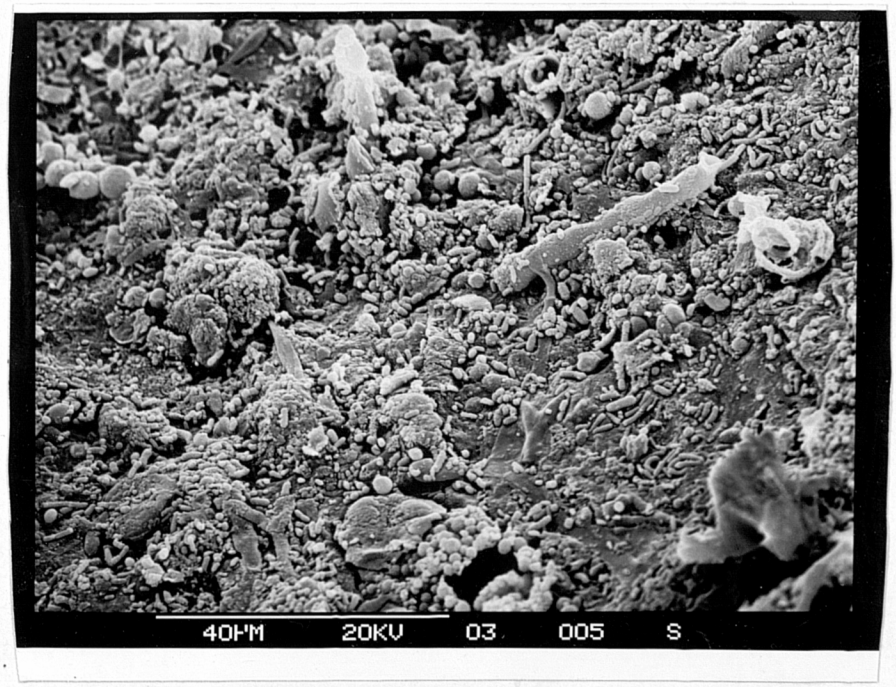


Figure 3b. Scanning electron micrograph of steeped grain showing dense coverage of bacteria and yeast/fungal spores on damaged area. Bar = 40um.

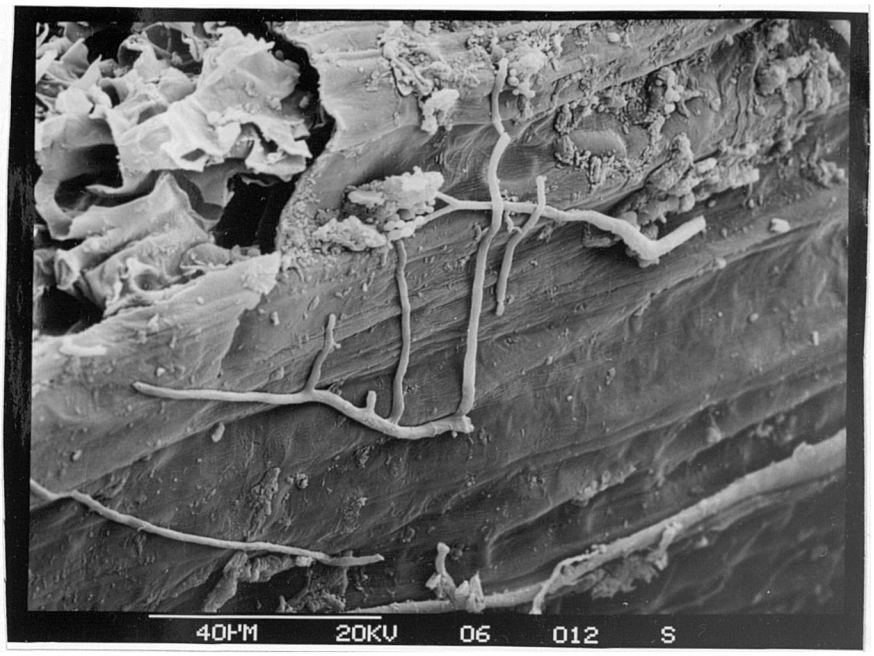


Figure 4. Scanning electron micrograph of kilned malt, with the husk removed, revealing the presence of branching fungal hyphae on the inner surface. Bar = 40um.

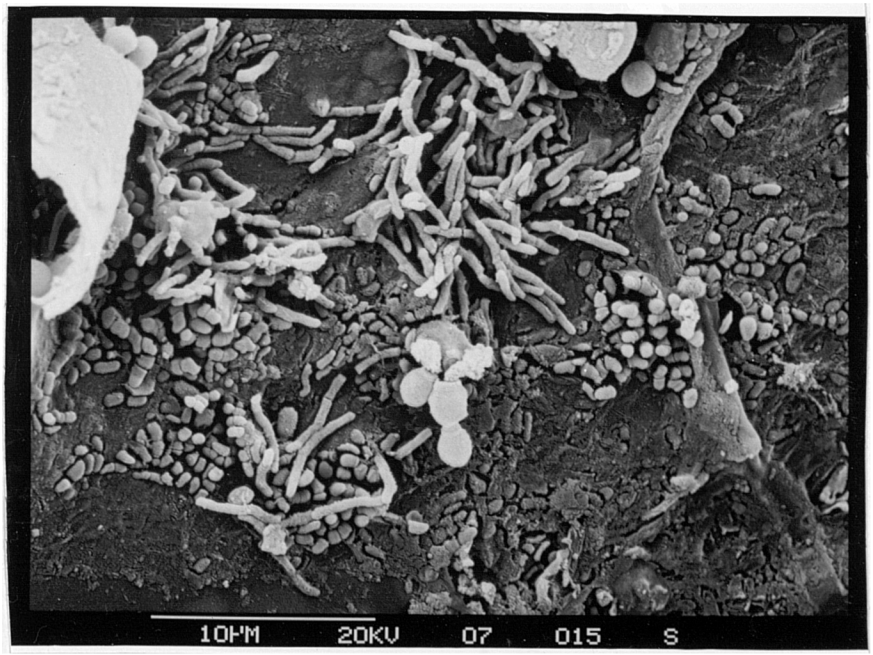


Figure 5. Micro-colonies of long bacteria in the presence of some fungal spores on the surface of the husk of kilned malt. Bar = 10um.

galactosidase, levan and oxidase production, degradation of casein, gelatin, starch and Tween 20 and 80, growth at 37° C and on 7.5% and 10% (w/v) sodium chloride, utilization of fructose, galactose, maltose, sucrose and xylose, and resistance to streptomycin, oxytetracycline, chloramphenicol, furazolidone, neomycin and penicillin, and 0.1 for haemolysis.

Clustering of the isolates:

The data for the isolates were analysed by the simple matching (S_{SM}) coefficient and clustering achieved using unweighted average linkage (UPMGA) methods. Dendrograms, based on the Jaccard (S_J) coefficient, were also examined before deciding membership of clusters. The results of the different computations gave broadly similar groupings but the interpretation of the data was based mainly on the S_{SM} coefficient insofar as it generated the most compact clusters.

At the 85% level of similarity, 345 (86%) of the aerobic heterotrophic bacteria and 4 (10%) of the reference cultures were recovered in 28 phena (Fig. 6). The characteristics of the phena are listed in Tables 4 and 5a, b and c.

Four reference strains aided the identification of phena 8, 11, 12 and 25 as Flavobacterium esteroaromaticum, Microbacterium lacticum, Arthrobacter globiformis and Pseudomonas putida, respectively. The characteristics of these groups were in broad agreement with the schemes in Starr et al. (1981) and Sneath et al. (1986). It should be emphasized, however, that Flav. esteroaromaticum is Gram-positive, and therefore not a bona fide representative of the genus Flavobacterium (Holmes et al., 1984). At present, its true taxonomic status is unclear. The remaining phena were identified as far as possible from

Figure 6. Simplified dendrogram, based on the S_{SM} Coefficient and UPGMA clustering algorithm, showing the relationship between clusters of aerobic heterotrophic bacteria recovered during barley malt production.

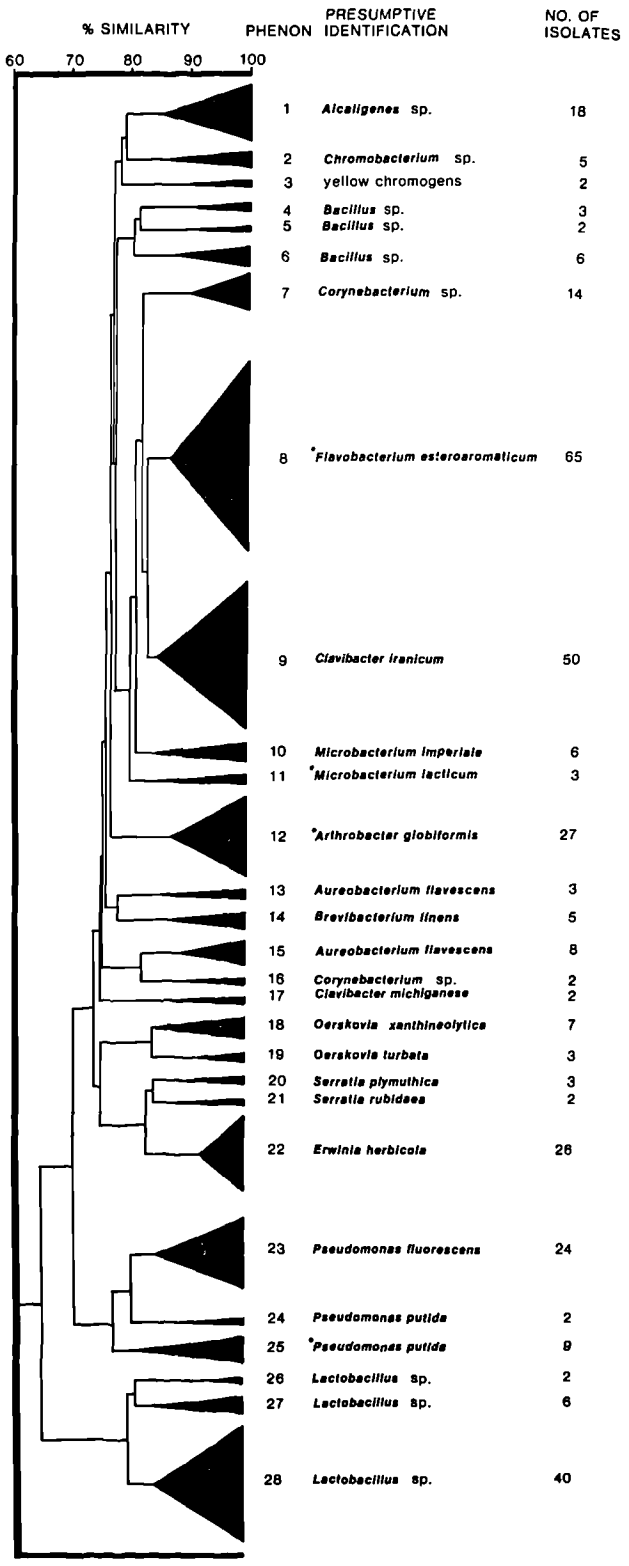

% SIMILARITY
60
70
80
90
100
PHENON
PRESUMPTIVE IDENTIFICATION
NO. OF ISOLATES
1 Alcaligenes sp. 18
2 Chromobacterium sp. 5
3 yellow chromogens 2
4 Bacillus sp. 3
5 Bacillus sp. 2
6 Bacillus sp. 6
7 Corynebacterium sp. 14
8 *Flavobacterium esteroaromaticum 65
9 Clavibacter iranicum 50
10 Microbacterium imperiale 6
11 *Microbacterium lacticum 3
12 *Arthrobacter globiformis 27
13 Aureobacterium flavescens 3
14 Brevibacterium linens 5
15 Aureobacterium flavescens 8
16 Corynebacterium sp. 2
17 Clavibacter michiganese 2
18 Oerskovia xanthineolytica 7
19 Oerskovia turbata 3
20 Serratia plymuthica 3
21 Serratia rubidaea 2
22 Erwinia herbicola 26
23 Pseudomonas fluorescens 24
24 Pseudomonas putida 2
25 *Pseudomonas putida 9
26 Lactobacillus sp. 2
27 Lactobacillus sp. 6
28 Lactobacillus sp. 40

Table 4

Characteristics of the major* phena given as % of positive reaction of unit characters

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
No. of strains tested:	18	65	50	27	26	24	40
Colonial characters:							
Levan produced	11	7	14	67	79	64	5
Colonies, white-cream	100	5	0	59	13	82	100
Colonies, yellow	0	95	60	41	88	14	0
Colonies, orange	0	0	38	0	0	0	0
Colonies, pink-red	0	0	0	0	0	0	0
Shiny/butyrous colonies	100	100	100	100	100	100	12
Colonies, matted	0	0	0	0	0	0	87
Colonies, round	100	100	100	100	100	100	100
Colonies, raised-entire	100	100	98	100	92	82	97
Diffusible pigment	6	0	0	0	0	14	0
Spreading colonies	11	0	0	0	0	4	0

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Colonial characters:							
Wrinkled colonies	0	0	0	0	0	4	0
Translucent colonies	0	2	0	0	0	4	2
"Gummy" colonies	0	8	40	7	0	0	0
Micro-colonies	6	0	0	0	0	0	95
Colonies buried in medium	0	0	22	0	0	0	0
Fluorescein produced	0	0	0	0	0	82	2
Stained preparations:							
Gram-positive	0	100	100	100	0	0	100
Gram-negative	100	0	0	0	100	100	0
Intracellular granules	11	23	16	4	8	27	7
Cocci	0	0	0	0	0	0	0
Short rods (<2 μm)	89	89	90	78	96	50	0
Medium rods (2 - 4 μm)	11	7	6	19	4	32	0
Long rods (4 - 5 μm)	0	0	0	0	0	4	100
Rounded ends	100	98	100	100	100	100	100

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Stained preparations:							
Acid fastness	0	0	0	0	0	0	0
Motility	100	80	0	56	79	68	62
Pleomorphism	0	3	4	4	0	18	0
Endospores	0	0	0	0	0	0	0
Anaerobiosis	100	100	100	10	100	0	100
Biochemical tests:							
β-galactosidase	44	80	60	41	100	41	92
Catalase	94	98	98	100	96	100	0
Oxidase	89	7	16	11	0	82	0
Fermentative metabolism	0	100	100	0	100	0	0
Oxidative metabolism	100	0	0	100	0	100	0
Gluconate oxidation	100	8	6	41	33	91	22
Lysine decarboxylase	0	7	26	0	0	0	85
Reduction, $NO_3 \rightarrow NO_2$	39	0	4	4	100	4	0

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Biochemical tests:							
Ornithine decarboxylase	6	2	18	0	0	0	97
Arginine dihydrolase	72	2	2	59	21	95	5
Methyl red	0	0	0	0	0	0	97
Voges Proskauer	6	0	0	0	72	0	87
H_2S production	6	2	2	4	4	0	0
Indole production	0	0	0	0	0	0	0
Phenylalanine deaminase	6	18	2	4	92	0	0
Tolerance to:							
MacConkey agar	94	98	80	96	96	100	92
pH 4	22	5	14	0	79	50	2
pH 5	100	97	96	100	100	100	100
pH 6	100	100	96	100	100	100	100
pH 8	100	100	98	100	100	100	100
pH 9	100	100	98	100	100	100	100

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Tolerance to:							
pH 10	100	100	98	100	100	100	100
NaCl (0% w/v)	100	100	100	100	100	100	100
NaCl (2.5% w/v)	100	100	100	100	100	100	90
NaCl (5% w/v)	83	97	0	100	100	100	12
NaCl (7.5% w/v)	28	16	0	93	71	36	2
NaCl (10% w/v)	0	0	0	63	42	0	2
CLED	100	98	98	100	96	100	97
Growth at:							
4° C	100	97	100	100	100	95	95
30° C	94	97	100	100	100	100	100
37° C	61	92	58	96	100	68	20
50° C	0	5	0	7	8	0	0

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Degradation of:							
DNA	0	93	66	100	33	54	10
Blood	83	92	64	4	72	82	2
Starch	94	100	18	100	83	0	10
Casein	94	90	92	100	100	100	57
Urea	83	12	2	100	13	100	5
Tyrosine	83	92	18	100	8	100	12
Aesculin	44	93	96	4	96	86	57
Gelatin	50	38	8	41	50	86	12
Lecithin	17	0	8	4	4	77	55
Elastin	0	2	0	0	0	9	0
Chitin	0	0	0	0	0	0	0
Tween 20	11	54	84	11	38	82	17
Tween 40	72	97	98	52	96	95	80
Tween 60	0	15	60	0	0	14	0

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Degradation of:							
Tween 80	0	54	74	4	25	91	2
Sole carbon source utilization:							
D-fructose	17	59	20	0	67	0	97
Lactose	0	15	2	0	21	0	92
D-glucose	61	7	0	0	100	45	92
Maltose	11	33	14	22	96	9	90
D-galactose	61	67	32	11	100	54	100
Sucrose	17	75	18	19	92	100	100
Glycerol	0	33	4	0	46	0	72
Ethanol	11	0	0	0	0	100	5
D-xylose	67	69	34	15	96	64	87
Sodium acetate	89	10	6	100	67	100	2
Sodium malonate	50	2	4	85	4	86	5
Sodium citrate	83	0	2	96	25	100	0

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Sole carbon source utilization:							
Sodium glutamate	83	5	10	85	92	91	12
Sodium tartrate	0	2	0	0	21	0	0
Calcium lactate	78	16	14	56	96	95	45
L-leucine	6	2	0	0	4	4	2
L-lysine	17	0	0	0	0	4	0
Resistance to:							
Streptomycin	0	2	0	0	0	4	0
Oxytetracycline	6	16	2	4	0	14	10
Chlortetracyline	0	5	0	0	4	14	10
Chloramphenicol	44	18	6	11	63	91	0
Furazolidone	83	64	84	89	29	95	7
Sulphafurazole	83	61	82	78	96	95	57
Neomycin	83	77	22	37	88	82	92

Table 4 (cont.)

	Phenon number and name						
	1	8	9	12	22	23	28
	Alcaligenes sp.	*Flavobacterium esteroaromaticum*	*Clavibacter iranicum*	*Arthrobacter globiformis*	*Erwinia herbicola*	*Pseudomonas fluorescens*	*Lactobacillus* sp.
Resistance to:							
Penicillin G	94	92	76	15	100	95	17

* = Positive characters common to all phenetic groups and excluded from this table was growth at 25° C and pH 7.

the conventional diagnostic schemes mentioned above.

Phenon 1 contained 18 strains that had the characteristics of the genus *Alcaligenes* (Kersters & De Ley, 1984). They were Gram-negative, obligately aerobic rods that showed positivity to oxidase, catalase production and negativity to indole production, and degraded starch but not DNA. However, it was not possible to identify the phenon up to species level.

Phenon 2, which comprised 5 strains, was identified as *Chromobacterium* sp. (Sneath, 1984). The strains were fermentative Gram-negative, motile, pigmented rods, with the ability to degrade aesculin, casein and elastin. They did not utilize xylose.

Table 5a

Characteristics of the minor phena*, given as % of positive reactions of unit characters.

	Phenon number						
	2	3	4	5	6	7	10
No. of strains:	5	2	3	3	6	14	6
Colonial characters:							
Levan produced	0	50	0	0	0	7	50
Colonies, white-cream	20	0	0	0	0	0	0
Colonies, yellow	0	100	0	0	0	100	17
Colonies, orange	0	0	100	100	100	0	83
Colonies, pink-red	80	0	0	0	0	0	0
Shiny colonies	100	100	100	100	100	100	100
Butyrous colonies	100	100	100	100	100	100	100
Colonies, matted	0	0	0	0	0	0	0
Colonies, round	100	100	100	100	100	100	100
Colonies, raised-entire	100	100	100	100	100	49	83
Colonies, granular	0	0	0	0	0	0	0
Diffusible pigment	20	0	0	0	0	14	0
Spreading colonies	0	0	0	0	0	0	0
Wrinkled colonies	0	0	0	0	0	0	0
Translucent colonies	0	0	0	0	0	0	0
"Gummy" colonies	0	0	0	0	17	70	33
Micro-colonies	0	0	0	0	0	0	0
Colonies buried in medium	0	0	0	0	17	14	0
Fluorescein produced	0	0	0	0	0	0	0

Table 5a (cont.)

	Phenon number						
	2	3	4	5	6	7	10
Stained preparations:							
Gram-positive	0	0	100	100	100	100	100
Gram-negative	100	100	0	0	0	0	0
Intracellular granules	20	0	0	50	0	0	17
Cocci	0	0	0	0	0	0	0
Short rods (<2 µm)	80	100	67	100	100	100	100
Medium rods (2 - 4 µm)	20	0	0	0	0	0	0
Long rods (4 - 5 µm)	0	0	0	0	0	0	0
Rounded ends	100	100	100	50	100	100	100
Acid fastness	0	0	0	0	0	0	0
Motility	100	100	67	50	33	21	50
Pleomorphism	0	0	33	50	0	0	0
Endospores	0	0	67	50	100	0	0
Anaerobiosis	100	100	100	100	100	100	10
Biochemical tests:							
ß-galactosidase	80	100	33	100	100	70	33
Catalase	80	100	100	50	100	100	100
Oxidase	60	0	0	50	33	0	0
Fermentative metabolism	100	0	100	100	33	0	33
Oxidative metabolism	0	0	0	0	0	89	33
Gluconate oxidation	0	100	33	0	17	0	0
Lysine decarboxylase	0	0	0	0	17	0	0
Reduction, NO_3--->NO_2	0	0	0	0	0	0	0

Table 5a (cont.)

	Phenon number						
	2	3	4	5	6	7	10
Biochemical tests:							
Ornithine decarboxylase	0	0	0	0	0	0	0
Arginine dihydrolase	20	50	0	0	0	0	0
Methyl red	0	0	0	0	0	0	0
Voges Proskauer	20	0	0	0	0	0	0
H_2S production	0	0	33	0	0	7	0
Indole production	0	0	0	0	0	0	0
Phenylalanine deaminase	20	0	0	0	0	84	17
Tolerance to:							
MacConkey agar	20	0	0	100	33	100	100
pH 4	0	0	0	50	0	56	50
pH 5	100	100	100	100	100	100	100
pH 6	100	100	100	100	100	100	100
pH 8	0	100	100	100	100	100	100
pH 9	0	100	100	100	100	100	100
pH 10	0	100	100	50	100	100	100
NaCl (0% w/v)	80	100	100	50	100	100	100
NaCl (2.5% w/v)	80	100	100	0	100	100	100
NaCl (5% w/v)	0	50	33	0	83	100	100
NaCl (7.5% w/v)	0	0	0	0	17	21	0
NaCl (10% w/v)	0	0	0	0	0	0	0
CLED	80	100	100	50	100	100	100

Table 5a (cont.)

	Phenon number						
	2	3	4	5	6	7	10
Growth at:							
4° C	100	100	100	100	100	100	100
30° C	100	100	100	0	67	100	100
37° C	80	100	33	50	50	89	33
50° C	20	0	0	50	0	0	50
Degradation of:							
DNA	20	100	33	50	0	100	100
Blood	80	100	100	100	67	100	100
Starch	100	100	100	50	100	100	10
Casein	100	100	33	50	50	84	50
Urea	20	0	0	0	0	7	0
Tyrosine	100	50	67	50	17	28	0
Aesculin	60	50	100	100	100	100	100
Gelatin	100	50	67	50	0	72	50
Lecithin	20	50	0	0	0	35	17
Elastin	100	0	0	0	0	0	0
Chitin	0	0	0	50	0	0	0
Tween 20	100	0	100	100	83	56	100
Tween 40	100	50	100	100	100	100	100
Tween 60	100	0	67	50	50	14	17
Tween 80	100	0	67	0	17	72	33
Sole carbon source utilization:							
D-fructose	0	0	0	50	0	84	17

Table 5a (cont.)

	Phenon number						
	2	3	4	5	6	7	10
Sole carbon source utilization:							
Lactose	0	0	0	0	0	28	0
D-glucose	0	0	0	0	17	0	0
Maltose	20	0	0	0	33	7	0
D-galactose	40	0	67	50	50	28	83
Sucrose	0	0	0	0	67	84	33
Glycerol	0	0	0	0	0	42	0
Ethanol	0	0	0	0	0	0	0
D-xylose	0	0	100	100	67	70	0
Sodium acetate	0	0	33	100	0	0	70
Sodium malonate	0	100	0	0	0	0	0
Sodium citrate	0	0	100	100	0	0	80
Sodium glutamate	20	100	33	100	17	21	17
Sodium tartrate	0	0	33	0	0	0	0
Calcium lactate	40	100	0	100	33	56	83
L-leucine	0	0	0	0	0	0	0
L-lysine	0	0	0	0	0	0	0
Resistance to:							
Streptomycin	80	50	0	50	17	0	17
Oxytetracycline	40	50	0	0	50	40	17
Chlortetracycline	0	50	33	0	50	7	0
Chloramphenicol	60	50	33	0	0	21	17
Furazolidone	20	100	100	50	50	84	33

Table 5a (cont.)

	Phenon number						
	2	3	4	5	6	7	10
Resistance to:							
Sulphafurazole	100	100	33	50	17	65	67
Neomycin	100	50	100	100	50	7	50
Penicillin G	100	100	100	50	83	84	100

* = Positive characters common to all phenetic groups and excluded from this table were growth at 25° C and pH 7.

Nevertheless, the group did not equate with any of the validly described species.

Unidentified Gram-negative, yellow chromogens with 2 strains, were recovered in phenon 3. These were motile, short rods, showing catalase production, degrading casein, blood, and starch; and utilizing sodium malonate, sodium glutamate and calcium lactate as sole carbon sources.

Small numbers of Gram-positive endospore-forming, facultatively anaerobic catalase-positive rods were recovered in phena 4, 5 and 6, with 3, 3 and 6 strains, respectively. These were orange-pigmented and were identified as Bacillus species. The distinguishing features of the phena are listed in Table 5a. However, they did not correspond to any species described by Claus & Berkeley (1986).

Gram-positive, non-endospore forming, mostly irregular shaped rods clustered in phena 7 to 19. Phena 7, 9, 16 and 17 with 14, 50, 2 and 2 isolates respectively, were considered to belong to coryneform

Table 5b

Characteristics of the minor phena*, given as % of positive reactions of unit characters.

	Phenon number						
	11	13	14	15	16	17	18
No. of strains:	3	3	5	8	2	2	7
Colonial characters:							
Levan produced	0	0	0	0	0	0	0
Colonies, white-cream	0	33	0	0	0	50	0
Colonies, yellow	100	67	80	100	50	0	100
Colonies, orange	0	0	20	0	50	0	0
Colonies, pink-red	0	0	0	0	0	50	0
Shiny colonies	100	100	80	100	100	100	100
Butyrous colonies	100	100	100	100	100	100	100
Colonies, matted	0	0	0	0	0	0	0
Colonies, round	100	100	100	100	100	100	100
Colonies, raised-entire	100	67	20	88	100	100	71
Colonies, granular	0	0	0	0	0	0	0
Diffusible pigment	0	0	0	0	0	0	0
Spreading colonies	0	0	0	0	0	0	0
Wrinkled colonies	0	0	0	0	0	0	0
Translucent colonies	0	100	20	0	0	0	0
"Gummy" colonies	33	33	80	13	0	0	0
Micro-colonies	0	0	0	0	0	0	0
Colonies buried in medium	0	67	60	0	0	0	0
Fluorescein produced	0	0	0	0	0	0	0

Table 5b (cont.)

	Phenon number						
	11	13	14	15	16	17	18
Stained preparations:							
Gram-positive	100	100	100	100	100	100	100
Gram-negative	0	0	0	0	0	0	0
Intracellular granules	0	0	0	75	0	100	57
Cocci	0	0	100	0	0	0	0
Short rods (<2 µm)	100	100	100	100	0	0	0
Medium rods (2 - 4 µm)	0	0	0	0	0	100	0
Long rods (4 - 5 µm)	0	0	0	0	50	0	100
Rounded ends	100	100	100	100	100	100	100
Acid fastness	0	0	0	0	0	0	0
Motility	33	33	0	38	0	0	57
Pleomorphism	0	33	10	13	50	60	0
Endospores	0	0	0	0	0	0	0
Anaerobiosis	10	10	10	10	100	100	100
Biochemical tests:							
ß-galactosidase	100	100	20	75	0	50	71
Catalase	100	100	100	100	100	100	100
Oxidase	67	0	0	0	0	50	0
Fermentative metabolism	100	0	0	0	0	0	100
Oxidative metabolism	0	67	100	100	100	50	0
Gluconate oxidation	0	0	0	25	0	0	0
Lysine decarboxylase	100	33	0	0	0	0	0
Reduction, NO_3--->NO_2	33	67	0	75	0	0	0

Table 5b (cont.)

	Phenon number						
	11	13	14	15	16	17	18
Biochemical tests:							
Ornithine decarboxylase	67	100	40	0	0	0	0
Arginine dihydrolase	0	0	0	0	0	0	0
Methyl red	33	0	0	0	0	0	0
Voges Proskauer	33	0	0	0	0	0	0
H_2S production	0	67	0	88	0	0	0
Indole production	0	0	0	0	0	0	0
Phenylalanine deaminase	0	0	0	13	0	0	57
Tolerance to:							
MacConkey agar	100	100	20	38	100	50	100
pH 4	0	67	20	25	0	0	29
pH 5	100	80	100	100	100	100	100
pH 6	100	100	100	100	100	100	100
pH 8	100	100	100	100	100	100	100
pH 9	100	100	100	100	100	100	100
pH 10	100	100	60	100	100	100	100
NaCl (0% w/v)	100	100	100	100	100	100	100
NaCl (2.5% w/v)	100	100	100	100	100	100	100
NaCl (5% w/v)	100	100	60	100	100	50	100
NaCl (7.5% w/v)	100	100	0	50	50	0	71
NaCl (10% w/v)	67	100	0	50	0	0	14
CLED	100	100	10	88	100	100	100

Table 5b (cont.)

	Phenon number						
	11	13	14	15	16	17	18
Growth at:							
4° C	100	100	100	100	100	100	100
30° C	100	100	100	100	100	50	100
37° C	100	100	40	75	100	0	71
50° C	0	33	40	0	0	0	14
Degradation of:							
DNA	100	100	0	75	50	100	13
Blood	100	100	80	100	100	100	86
Starch	100	100	80	75	100	100	71
Casein	67	100	80	38	100	100	29
Urea	21	0	0	0	100	0	0
Tyrosine	67	0	0	13	50	0	14
Aesculin	100	100	80	38	100	100	100
Gelatin	67	33	20	50	50	0	71
Lecithin	33	67	20	0	0	0	0
Elastin	0	0	0	0	0	0	0
Chitin	0	0	0	0	0	0	0
Tween 20	0	100	100	100	100	100	14
Tween 40	67	100	100	100	100	100	100
Tween 60	0	0	60	100	50	100	14
Tween 80	67	100	100	100	100	100	0
Sole carbon source utilization:							
D-fructose	100	0	0	0	0	0	54

Table 5b (cont.)

	Phenon number						
	11	13	14	15	16	17	18
Sole carbon source utilization:							
Lactose	0	0	0	0	0	0	0
D-glucose	33	0	0	0	0	0	71
Maltose	33	0	0	13	0	100	14
D-galactose	67	33	80	0	0	50	14
Sucrose	67	0	40	0	0	100	71
Glycerol	100	0	0	0	0	100	14
Ethanol	0	0	0	0	0	0	0
D-xylose	33	67	60	0	0	100	43
Sodium acetate	80	0	0	100	100	0	14
Sodium malonate	0	0	0	13	100	0	0
Sodium citrate	80	0	0	100	100	0	0
Sodium glutamate	0	0	0	0	50	0	0
Sodium tartrate	0	0	0	0	0	0	14
Calcium lactate	70	33	20	25	100	0	14
L-leucine	0	0	0	0	0	0	0
L-lysine	0	0	0	0	0	0	0
Resistance to:							
Streptomycin	0	0	0	0	0	0	0
Oxytetracycline	0	0	0	0	0	0	0
Chlortetracycline	0	0	0	13	0	0	14
Chloramphenicol	0	100	0	38	50	0	43
Furazolidone	33	100	60	88	100	0	86

Table 5b (cont.)

	Phenon number						
	11	13	14	15	16	17	18
Resistance to:							
Sulphafurazole	67	100	80	75	100	0	71
Neomycin	0	0	0	38	50	0	57
Penicillin G	33	100	40	75	100	50	100

* = Positive characters common to all phenetic groups and excluded from this table were growth at 25° C and pH 7.

bacteria, with similarities to the genera Corynebacterium and Clavibacter (Collins & Cummins, 1986). In particular, phena 9 and 17 approximated with descriptions of plant pathogenic taxa, namely Clavibacter iranicum and Cla. michiganese, respectively. Thus, the organisms in phenon 9 comprised yellow/orange-pigmented, facultatively anaerobic, non-motile, non-acid fast, short rods, which grew in a maximum of 2.5% (w/v) sodium chloride, were negative for the methyl red test, and did not degrade gelatin or starch, or utilize acetate or lactate (Collins & Bradbury, 1986). Phenon 17 comprised small pleomorphic rods with intracellular granules, which were catalase positive, and demonstrated a respiratory metabolism. Six strains, clustered in phenon 10, were Gram-positive small irregular rods which were considered as Microbacterium imperiale (Collins & Keddie, 1986). These yellow/orange-pigmented, motile organisms did not reduce nitrate, produce H_2S or degrade starch or urea, but acetate, lactate and citrate

Table 5c

Characteristics of the minor phena*, given as % of positive reactions of unit characters.

	Phenon number						
	19	20	21	24	25	26	27
No. of strains:	3	3	2	2	9	2	6
Colonial characters:							
Levan produced	0	100	50	0	0	0	67
Colonies, white-cream	0	100	0	100	100	100	100
Colonies, yellow	100	0	0	0	0	0	0
Colonies, orange	0	0	0	0	0	0	0
Colonies, pink-red	0	0	100	0	0	0	0
Shiny colonies	100	100	100	50	100	0	0
Butyrous colonies	100	100	100	50	100	0	0
Colonies, matted	0	0	0	50	0	100	100
Colonies, round	100	100	100	100	89	100	100
Colonies, raised-entire	100	100	100	100	100	100	100
Colonies, granular	0	0	0	0	0	0	0
Diffusible pigment	0	0	0	0	11	0	0
Spreading colonies	0	0	0	0	0	0	0
Wrinkled colonies	0	0	0	0	0	0	0
Translucent colonies	0	0	0	0	0	50	33
"Gummy" colonies	33	0	0	0	0	0	0
Micro-colonies	0	0	0	0	0	100	83
Colonies buried in medium	0	0	0	0	0	0	0
Fluorescein produced	0	0	0	50	22	0	0

Table 5c (cont.)

	Phenon number						
	19	20	21	24	25	26	27
Stained preparations:							
Gram-positive	100	0	0	0	0	100	100
Gram-negative	0	100	100	100	100	0	0
Intracellular granules	67	0	50	50	0	100	0
Cocci	0	0	0	0	0	0	17
Short rods (<2 µm)	0	100	50	0	78	0	0
Medium rods (2 - 4 µm)	0	0	50	100	0	0	0
Long rods (4 - 5 µm)	100	0	0	0	11	100	100
Rounded ends	100	100	100	100	100	100	83
Acid fastness	0	0	0	0	0	0	0
Motility	33	100	100	50	100	100	33
Pleomorphism	0	0	0	0	11	0	0
Endospores	0	0	0	0	0	0	0
Anaerobiosis	100	100	100	0	0	100	100
Biochemical tests:							
ß-galactosidase	67	100	100	100	44	50	67
Catalase	100	100	100	100	100	0	0
Oxidase	27	0	0	50	100	0	0
Fermentative metabolism	100	100	100	0	0	0	0
Oxidative metabolism	0	0	0	100	100	0	0
Gluconate oxidation	0	67	0	100	67	0	0
Lysine decarboxylase	100	0	0	0	0	100	83
Reduction, NO_3--->NO_2	0	100	50	100	22	0	17

Table 5c (cont.)

	Phenon number						
	19	20	21	24	25	26	27
Biochemical tests:							
Ornithine decarboxylase	100	0	0	0	0	100	100
Arginine dihydrolase	0	67	100	100	89	0	0
Methyl red	100	0	0	0	0	50	83
Voges Proskauer	0	100	100	0	0	0	0
H_2S production	0	0	0	0	0	0	0
Indole production	0	0	0	0	0	0	0
Phenylalanine deaminase	0	33	0	0	0	0	0
Tolerance to:							
MacConkey agar	100	100	100	100	100	0	33
pH 4	0	67	50	100	22	0	0
pH 5	100	100	100	100	100	100	100
pH 6	100	100	100	100	100	100	100
pH 8	100	100	100	100	100	100	100
pH 9	100	100	100	100	100	100	100
pH 10	100	100	100	100	100	0	67
NaCl (0% w/v)	100	100	100	100	100	100	100
NaCl (2.5% w/v)	100	100	100	100	100	100	100
NaCl (5% w/v)	100	100	100	100	100	0	100
NaCl (7.5% w/v)	67	33	50	0	11	0	50
NaCl (10% w/v)	0	0	0	0	0	0	0
CLED	100	100	100	100	100	100	67

Table 5c (cont.)

	Phenon number						
	19	20	21	24	25	26	27
Growth at:							
4° C	100	100	0	100	100	100	100
30° C	100	100	100	100	100	100	100
37° C	100	67	100	100	100	50	83
50° C	0	0	0	10	11	0	17
Degradation of:							
DNA	33	0	0	100	0	0	0
Blood	33	0	50	100	11	0	17
Starch	100	100	100	0	0	0	0
Casein	67	100	0	100	89	50	50
Urea	67	67	50	100	100	0	0
Tyrosine	0	10	50	100	100	0	17
Aesculin	67	100	100	100	22	0	100
Gelatin	100	100	100	0	0	50	0
Lecithin	67	0	0	0	11	50	17
Elastin	0	0	0	0	0	0	0
Chitin	0	100	0	0	0	0	0
Tween 20	0	33	0	100	22	0	0
Tween 40	100	67	0	100	67	100	100
Tween 60	0	33	0	0	11	0	0
Tween 80	0	33	100	0	30	0	0
Sole carbon source utilization:							
D-fructose	100	67	50	100	11	50	100

Table 5c (cont.)

	Phenon number						
	19	20	21	24	25	26	27
Sole carbon source utilization:							
Lactose	33	100	100	0	0	0	0
D-glucose	100	67	100	100	67	100	100
Maltose	0	67	100	100	44	100	83
D-galactose	0	100	100	100	33	50	83
Sucrose	100	67	100	100	11	100	100
Glycerol	0	67	100	0	0	0	0
Ethanol	0	0	100	0	22	0	0
D-xylose	100	100	0	100	78	100	83
Sodium acetate	67	67	100	100	100	0	0
Sodium malonate	0	67	50	100	67	0	0
Sodium citrate	33	100	100	100	100	0	0
Sodium glutamate	0	100	100	100	100	0	17
Sodium tartrate	0	0	0	0	11	0	0
Calcium lactate	33	100	50	100	78	0	17
L-leucine	0	0	0	0	56	0	0
L-lysine	0	0	0	0	0	0	0
Resistance to:							
Streptomycin	0	33	0	0	78	50	33
Oxytetracycline	0	33	0	0	44	0	0
Chlortetracycline	0	33	0	0	33	0	17
Chloramphenicol	0	100	100	100	100	50	17
Furazolidone	100	0	0	100	100	100	83

Table 5c (cont.)

	Phenon number						
	19	20	21	24	25	26	27
Resistance to:							
Sulphafurazole	100	100	100	100	100	100	100
Neomycin	0	100	50	100	100	0	67
Penicillin G	33	100	100	100	100	50	33

* = Positive characters common to all phenetic groups and excluded from this table were growth at 25° C and pH 7.

were utilized as sole sources of carbon and energy. Half of the isolates degraded gelatin weakly. Both phena 13 and 15 which contained 3 and 8 isolates, respectively were presumptively identified as Aureobacterium flavescens from their key characteristics (Komagata & Suzuki, 1986). Essentially, these were yellow-pigmented short irregular shaped, obligately aerobic, catalase-positive rods, which were Gram-positive, hydrolysed starch, DNA and gelatin, did not produce urease but reduced nitrate to nitrite. Phenon 14, which comprised 5 strains of Gram-positive obligately aerobic rods and cocci, resembled Brevibacterium linens (Jones & Keddie, 1986). These organisms produced bright yellow/orange colonies, and did not produce oxidase. A positive response was recorded to the colony colour reaction of Jones et al. (1973). Facultatively anaerobic Gram-positive motile rods which displayed extensive branching but without production of aerial mycelia, were identified as Oerskovia (Lechevalier & Lechevalier, 1986). Thus

phena 18 and 19 with 7 and 3 strains, respectively, were identified as O. xanthineolytica and O. turbata, respectively. These phena were differentiated from each other on the basis of growth at 42° C, and for which phenon 18 gave a positive response, also including temperatures above 42° C, although Sottnek et al. (1977) considered this parameter as variable.

Gram-negative, fermentative, catalase positive but oxidase negative rods, which met the requirements for the family Enterobacteriaceae (Brenner, 1984) were recovered in phena 20 to 22. Phenon 20, which contained 3 strains, approximated to the description of Serratia plymuthica (Grimont & Grimont, 1984). The organisms formed off-white colonies on PCA, which contained highly motile cells. These exhibited good growth at 4° C. Chitin and Tween 80 were attacked, but neither lysine nor ornithine are decarboxylated. In contrast, phenon 21 with 2 strains, which was identified as Ser. rubidaea (Grimont & Grimont, 1984), contained red-pigmented organisms. These did not grow at 4° C, attacked Tween 80 but not chitin, and did not decarboxylate lysine or ornithine. Phenon 22, which comprised 26 strains of yellow-pigmented motile organisms, was equated with Erwinia herbicola (Lelliott & Dickey, 1984), although the reference culture, ie. NCPPB 656, was not included in this group. Isolates degraded gelatin, produced phenylalanine deaminase but not indole, reduced nitrate and grew in 5% (w/v) sodium chloride.

Motile, oxidative, Gram-negative, oxidase and catalase positive rods, producing diffusible fluorescent pigments were recovered in phena 23 to 25, and identified as representatives of the genus Pseudomonas. Phenon 23, with 24 strains, equated with the description of Ps. fluorescens (Palleroni, 1984), despite the recovery elsewhere of the

reference strain. Cultures produced levan and arginine dihydrolase, and grew at 4° C. Gelatin, lecithin and Tween 80 but not starch were hydrolysed. Ethanol and sucrose were utilized as the sole source of carbon and energy. Indeed, phenon 23 approximated the characteristics of Ps. fluorescens biovar I. Phenon 24, with only 2 strains, was closest to the characteristics of Ps. putida biovar B (Palleroni, 1984). In particular, the isolates produced arginine dihydrolase but not levan, and did not degrade gelatin, lecithin, starch or Tween 80.

Phena 26 to 28, with 2, 6 and 40 strains, respectively, comprised long non-spore forming rods, which were tentatively included in the genus Lactobacillus, although it was not possible to identify the organisms to the species level (Kandler & Weiss, 1986). Cultures were off-white and produced Gram-positive cells.

After the completion of the computer analysis differential characteristics of all 28 phena, which should permit the identification of fresh strains, have been constructed (Table 6). This includes tests that are simple to carry out routinely in the laboratory.

Lactobacilli

Besides the lactobacilli included in the numerical taxonomy study, cultures were also isolated on selective medium, i.e. MRS agar. These organisms were identified as L. acidophilus, L. alimentarius and L. plantarum which accounted for 9, 59 and 32% of the strains, respectively. The characteristics of the lactobacilli are shown in Table 7. Essentially, all strains produced white colonies which contained catalase negative, non-motile, non-endospore forming, facultatively anaerobic, Gram-positive rods. Moreover, neither casein, gelatin nor starch was degraded, nor was H_2S or NH_3 (from arginine)

Table 6

Differential characteristics of the aerobic heterotrophic bacteria

Phenon	Colony pigmentation	Gram positivity	Oxidative (O)/fermentative (F) metabolism	Gluconate oxidation	Oxidase production	Phenyalanine deaminase	Lysine decarboxylase	Degradation of: Aesculin	Blood	Casein	DNA	Tween 20	Tween 80	Tyrosine	Urea	Utilization of calcium lactate	Utilization of sucrose	Growth at 50° C
1	W	-	O	+	+	-	-	V	+	+	-	-	-	+	+	V	-	-
2	P	-	F	-	V	-	-	V	+	+	-	+	+	+	-	V	-	-
3	Y	-	-	+	-	-	-	V	+	+	+	-	-	V	-	+	-	-
4	Or	+	F	V	-	-	-	+	+	V	V	+	V	V	-	-	-	-
5	Or	+	F	-	V	-	-	+	+	V	V	+	-	V	-	+	-	V
6	Or	+	F	-	V	-	-	+	V	V	-	+	-	-	-	V	V	-
7	Y	+	O	-	-	+	-	+	+	+	+	V	V	V	-	V	+	-
8	Y	+	F	-	-	-	-	+	+	+	+	V	V	+	-	-	V	-
9	Y/Or	+	F	-	-	-	V	+	V	+	V	+	V	-	-	-	-	-
10	Y/Or	+	Gr	-	-	-	-	+	+	V	+	+	V	-	-	+	V	V
11	Y	+	F	-	V	-	+	+	+	V	+	-	V	V	V	V	V	-
12	W/Y	+	O	V	-	-	-	-	-	+	+	-	-	+	+	V	-	-
13	W/Y	+	O	-	-	-	V	+	+	+	+	+	+	-	-	V	-	V
14	Y/Or	+	O	-	+	-	-	+	+	+	-	+	+	-	-	-	V	V

Table 6 (cont.)

Phenon	Colony pigmentation	Gram positivity	Oxidative (O)/fermentative (F) metabolism	Gluconate oxidation	Oxidase production	Phenylalanine deaminase	Lysine decarboxylase	Degradation of: Aesculin	Blood	Casein	DNA	Tween 20	Tween 80	Tyrosine	Urea	Utilization of calcium lactate	Utilization of sucrose	Growth at 50° C
15	Y	+	O	V	–	–	–	V	+	V	V	+	+	–	+	V	–	–
16	Y/Or	+	O	–	–	–	–	+	+	+	V	+	+	V	+	+	–	–
17	W/P	+	O	–	V	–	–	+	+	+	+	+	+	–	–	–	+	–
18	Y	+	F	–	–	V	–	+	+	V	–	–	–	–	–	–	V	–
19	Y	+	F	–	V	–	+	V	V	V	V	–	–	–	V	V	+	–
20	W	–	F	V	V	V	–	+	–	+	–	V	V	–	V	+	V	–
21	P	–	F	–	–	–	–	+	V	–	–	–	–	–	V	V	+	–
22	W/Y	–	F	V	–	+	–	+	V	+	V	V	V	–	–	+	+	–
23	W/Y	–	O	+	+	–	–	+	+	+	V	+	+	+	+	+	–	–
24	W	–	O	+	V	–	–	+	+	+	+	+	+	+	+	+	+	+
25	W	–	O	V	+	–	–	V	–	+	–	V	V	+	+	V	–	–
26	W	+	Gr	–	–	–	+	–	–	V	–	–	–	–	–	–	+	–
27	W	+	Gr	–	–	–	+	+	–	V	–	–	–	–	–	–	+	–
28	W	+	Gr	V	–	–	+	V	–	V	–	–	–	–	–	V	+	–

+, - and V correspond to >80, <20 and 21 - 79% of positive responses, respectively.

W, Or, Y and P correspond to white, orange, yellow and pink, respectively.

Gr = growth.

produced. Growth occurred at 15° C. Cellobiose, fructose, maltose, mannose and trehalose, but not rhamnose, were fermented. Strains, identified as L. acidophilus, fermented amygdalin, galactose, aesculin, lactose, salicin and sucrose, but not ribose, sorbitol or xylose. Neither ethanol, mannitol nor sodium citrate were utilized. These characteristics were in good accord with the description by Kandler & Weiss (1986). Similarly, the characteristics of L. alimentarius matched the species description, insofar as strains fermented aesculin, amygdalin, arabinose, galactose, gluconate, melezitose, sorbitol, salicin and sucrose, but not lactose, melibiose, raffinose or xylose (Kandler & Weiss, 1986). The strains of L. plantarum fermented amygdalin, aesculin, arabinose, galactose, glucose, gluconate, lactose, melezitose, melibiose, raffinose, salicin, sucrose, ribose and xylose. In contrast to the species description, mannitol was not fermented (Kandler & Weiss, 1986). However, mannitol is not a key character.

DISTRIBUTION OF BACTERIA AMONG SAMPLES

Distribution (%) of the phenetic groups among the environmental samples is given in Table 8.

Table 7

Characteristics of presumptive lactobacilli isolated from malting barley.

Character	Presumptive identification: *Lactobacillus alimentarius*	*L. plantarum*	*L. acidophilus*
Isolate number:	13	7	2
Colonial characteristics:			
Size (2 - 5 um)	+	+	+
Entire margin	+	+	+
Convex	+	+	+
Opaque	+	+	+
White colour	+	+	+
Pellicle	-	-	-
Micro-morphology:			
Rod shaped cells	+	+	+
Gram reaction	+	+	+
Motility	-	-	-
Spore formation	-	-	-
Growth at 15° C	+	+	+
Growth at 45° C	-	-	+
Anaerobiosis	+	+	+
Biochemical characteristics:			
Catalase production	-	-	-
Rhamnose	-	-	-
Amygdalin	+	+	+

Table 7 (cont.)

Character	Presumptive identification: *Lactobacillus alimentarius*	*L. plantarum*	*L. acidophilus*
Biochemical characteristics:			
L-Arabinose	+	+	+
Cellobiose	+	+	+
Aesculin	+	+	+
D-Fructose	+	+	+
D-Galactose	+	+	+
D-Glucose	+	+	+
Gluconate	+	+	d
Lactose	-	+	+
Maltose	+	+	+
Mannitol	d	-	-
Mannose	+	+	+
Melezitose	d	+	-
Melibiose	-	+	d
Raffinose	-	+	d
D-Ribose	d	+	-
Sorbitol	+	+	-
Salicin	+	d	d
Trehalose	+	+	+
D-Xylose	-	+	-
Sucrose	+	+	+
Degradation of:			
Casein	-	-	-

Table 7 (cont.)

Character	Presumptive identification: *Lactobacillus alimentarius*	*L. plantarum*	*L. acidophilus*
Degradation of:			
Starch	-	-	-
Ethanol	-	-	-
Sodium citrate	d	-	-
Malic acid	-	-	-
Gelatin	-	-	-
H_2S production	-	-	-
Levan production	d	d	d
NH_3 from arginine	-	-	-

+ = positive, >90%; d = weakly positive, 10 - 80%; - = negative, <10%.

Dried barley

A diverse bacterial microflora, comprising mostly Gram-positive pigmented organisms, were present on dried barley kernels. Indeed, representatives of 13 taxa were present, including *Aureobacterium flavescens* (phena 13 and 15), *Bacillus* sp. (phenon 4), *Brevibacterium linens* (phenon 14), *Corynebacterium* spp. (phena 7 and 16), *Clavibacter iranicum* (phenon 9), *Microbacterium imperiale* (phenon 10), *Oerskovia xanthineolytica* (phenon 18), *Erwinia herbicola* (phenon 22), *Pseudomonas*

Table 8

Distribution (%) of the phenetic groups among the environmental samples.

Phenon	Number of isolates	Sample type: Dry barley	Water (first steep)	Barley (first steep)	Water (second steep)	Barley (second steep)	Green malt	Kilned malt	Screened malt
1	18	0	5	11	50	33	0	0	0
2	5	20	0	20	0	40	20	0	0
3	2	50	0	0	0	0	50	0	0
4	3	33	33	0	0	33	0	0	0
5	2	0	50	0	50	0	0	0	0
6	6	0	0	0	33	17	33	17	0
7	14	64	0	0	28	7	0	0	0
8	65	0	0	25	8	13	20	21	13
9	50	4	0	8	0	16	4	30	38
10	6	32	17	17	17	17	0	0	0
11	3	0	33	0	0	0	0	33	33
12	27	0	0	0	0	0	18	41	41
13	3	100	0	0	0	0	0	0	0
14	5	80	0	0	20	0	0	0	0

Table 8 (cont.).

Phenon	Number of isolates	Sample type: Dry barley	Water (first steep)	Barley (first steep)	Water (second steep)	Barley(second steep)	Green malt	Kilned malt	Screened malt
15	8	38	0	25	0	0	25	0	12
16	2	50	0	0	0	50	0	0	0
17	2	0	0	100	0	0	0	0	0
18	7	43	0	0	0	14	29	14	0
19	3	0	0	0	0	0	0	0	100
20	3	0	0	0	0	0	100	0	0
21	2	0	0	0	0	0	0	0	100
22	26	8	16	24	12	12	12	8	8
23	24	32	16	28	8	8	8	0	0
24	2	0	0	100	0	0	0	0	0
25	9	0	0	0	0	0	100	0	0
26	2	0	50	0	50	0	0	0	0
27	6	0	17	0	50	0	17	17	0
28	40	0	65	0	25	8	2	0	0

fluorescens (phenon 23), Chromobacterium sp. (phenon 2) and unidentified yellow chromogens (phenon 3), together with 10 single member clusters. In fact, all of the isolates of Aur. flavescens (phenon 13) and most of the strains of Brev. linens (phenon 14) were derived from dry barley. Although there was not a noticeable dominance of any taxa, the populations comprised Corynebacterium sp. (phenon 7; 18% of the total number of isolates from dried barley) and Ps. fluorescens (phenon 23; 16% of the total number of isolates from dried barley).

Barley (after first steep)

Compared to dried barley, a narrower range of taxa was present on the first steeped kernels. These bacteria were identified as Aureobacterium flavescens (phenon 15), Alcaligenes (phenon 1), Chromobacterium sp. (phenon 2), Cla. iranicum (phenon 9), all the isolates of Cla. michiganese (phenon 17), Erwinia herbicola (phenon 22), Fla. esteroaromaticum (phenon 8), M. imperiale (phenon 10), Ps. fluorescens (phenon 23) and 100% of the isolates of Ps. putida (phenon 24). Again, Gram-positive chromogens dominated, with the largest number of isolates (32% of the total recovered from first steeped barley) comprising Fla. esteroaromaticum. This taxon assumingly contributed to the increased number of aerobic heterotrophic bacteria recovered on this sample type.

Second steeped barley

The range of bacterial taxa increased substantially. Representatives of 13 taxa most of which comprised Gram-positive rods, together with 11 single member clusters were recovered. However, there

was not any dominant organism. It is noteworthy that lactobacilli appeared in substantial numbers. Indeed, all of the representatives of L. acidophilus recovered on MRS medium were from this sample type. The 13 taxa included Alcaligenes sp. (phenon 1), Chromobacterium sp. (phenon 2), Bacillus sp. (phena 4, 6), Corynebacterium sp. (phena 7, 16), Fla. esteroaromaticum (phenon 8), Clavibacter iranicum (phenon 9), Microbacterium imperiale (phenon 10), Oerskovia xanthineolytica (phenon 18), Erwinia herbicola (phenon 22), Ps. fluorescens (phenon 23), and Lactobacillus sp. (phenon 28).

Green malt

With germination, there was an enhanced number of taxa on green malt, comprising Gram-negative bacteria, including Chromobacterium (phenon 2), Erw. herbicola (phenon 22), Ps. fluorescens (phenon 23), all the strains of Ps. putida (phenon 25) and Serratia plymuthica (phenon 20). The dominant taxon, in terms of numbers of isolates recovered, was Fla. esteroaromaticum (26% of the total strains from green malt). Undoubtedly, this contributed to the greatly increased numbers of bacteria on green malt. Lactobacillus increased in numerical importance, with representatives equated with Lactobacillus sp. (phena 27 and 28), and L. alimentarius and L. plantarum. Fourteen phena were recovered in this sample type.

Kilned malt

With kilning, the overall microbial numbers and the range of bacterial taxa declined. Six groups, containing Gram-positive organisms were recovered together with only one representative of a

Gram-negative taxon, i.e. Erw. herbicola. The dominant groups were Cla. iranicum (phenon 9; 30% of the isolates from the sample), Fla. esteroaromaticum (28% of the isolates from the sample) and Arthrobacter globiformis (phenon 12, 24% of the total), the latter of which made its first appearance in this sample. Most of the lactobacilli were identified as L. alimentarius.

Screened malt

A low population diversity was also apparent on screened malt. Here, the majority of the taxa were Gram-positive, including Arth. globiformis (phenon 12), Aur. flavescens (phenon 15), Cla. iranicum (phenon 9; this was the dominant taxon, accounting for 38% of the strains from the sample), Fla. esteroaromaticum (phenon 8), Mic. lacticum (phenon 11) and 100% of the isolates of Oerskovi turbata (phenon 19). The lactobacilli were identified as L. alimentarius and L. plantarum. The Gram-negative representatives of the microflora were equated with Erw. herbicola (phenon 22) and all of the strains of Serratia rubidaea.

Bore-hole, first and second steep-water

From initial small populations of cream-coloured aeromonads, lactobacilli and pseudomonads in bore-hole water, there was proliferation of overall numbers in the first steep water. Here, the taxonomic composition of the microflora reflected some of the types present on the barley. The dominant taxon was Lactobacillus (phenon 28; 52% of the total number of isolates for the sample), with lower numbers of other lactobacilli, Alcaligenes sp. (phenon 1), Bacillus sp. (phena 4 and 5), Erw. herbicola (phenon 22), Mic. imperiale (phenon

10), Mic. lacticum (phenon 11) and Ps. fluorescens (phenon 23). The higher microbial counts in the second steep water did not reflect the numerical dominance of any particular taxon. In this sample, the bacteria were equated with Alcaligenes sp. (phenon 1), Bacillus sp. (phena 5 and 6), Brev. linens (phenon 14), Corynebacterium sp. (phenon 7), Erw. herbicola (phenon 22), Fla. esteroaromaticum (phenon 8), Mic. imperiale (phenon 10), Ps. fluorescens (phenon 23) and Lactobacillus sp. (phena 26, 27 and 28; Lac. plantarum).

Examination of the overall distribution of the bacterial groups revealed that 7 phena were restricted to one given sample type. Thus, Aur. flavescens (phenon 13) was restricted to dried barley, Cla. michiganese (phenon 17) and Ps. putida (phenon 25) and Ser. plymuthica (phenon 20) to green malt, and O. turbata (phenon 19) and Ser. rubidaea (phenon 21) to the screened malt. Only Erw. herbicola was common to all sample types. Others were restricted to some samples. For example, Bacillus sp. (phenon 5) and Lactobacillus sp. (phenon 26) occurred in water (first and second steep). Alcaligenes sp. (phenon 1) was recovered from first and second steep water and barley. Pseudomonas fluorescens (phenon 24) occurred universally until kilning, when it effectively disappeared, perhaps indicating the inability of the taxon to survive the heating process.

Filamentous Fungi

The 96 filamentous fungi isolates (including 47 from direct plating) were examined, identified and the results are shown in Tables 9 and 10.

From the dilution plates, 18 fungal species were recovered, comprising 12 field fungi and 6 storage fungi (Table 9). Examination

Table 9

Filamentous fungi detected at stages during malting of barley on PDA at 25° C, using dilution plating technique.

Filamentous fungi	Stage of malting								
	Dried barley	First steep grain	Second steep grain	Green malt	Kilned malt	Screened malt	First steep water	Second steep water	Bore-hole water
Field fungi									
Aureobasidium pullulans	-	-	-	-	-	-	-	+	-
Chrysosporium sp.	-	-	-	-	-	-	+	-	-
Cladosporium cladosporioides	+	-	+	-	-	+	+	+	-
C. macrocarpum	-	-	-	-	-	-	-	+	-
Fusarium avenaceum	-	-	+	+	-	+	+	+	-
F. dimerum	-	-	-	-	-	-	+	-	-
F. lateritium	-	-	-	-	+	-	-	-	-
F. nivale	-	+	-	-	-	-	-	-	-
F. poae	-	-	-	-	-	+	-	+	-
Geotrichum candidum	-	-	-	+	+	+	-	-	-
Phoma herbarum	-	-	-	-	+	-	-	+	-
Verticillium lecanii	-	-	-	-	-	-	-	+	-

Table 9 (cont.)

Filamentous Fungi	Stage of malting								
	Dried barley	First steep grain	Second steep grain	Green malt	Kilned malt	Screened malt	First steep water	Second steep water	Bore-hole water
Storage fungi									
Aspergillus clavatus	+	+	+	-	-	-	+	+	-
A. flavus	+	-	-	-	-	-	-	-	-
Mucor hiemalis	-	-	-	-	-	+	-	-	-
Penicillium brevicompactum	-	-	-	-	-	-	+	-	-
P. chrysogenum	+	+	-	-	-	-	+	-	-
Penicillium sp.	-	-	-	-	-	+	+	-	-

+ = present; - = absent.

Table 10

Frequency of dry barley and screened malt kernels contaminated by different categories of filamentous fungi (a), and MFI values (b); values computed from results of direct plating on MSA and PDA at 25° C.

Isolate	Percentage frequency: Dry barley	Screened malt
(a)		
Field Fungi		
Alternaria alternata	46	16
Aureobasidium pullulans	1	0
Botrytis cinerea	13	2
Cladosporium cladosporiodes	14	0
Cochliobolus sativus	1	0
Epicoccum purpurascens	22	6
Fusarium avenaceum	38	23
Geotrichum candidum	0	21
Phoma herbarum	1	1
Pyrenophora teres	1	1
Storage Fungi		
Absidia corymbifera	3	12
Aspergillus candidus	3	2
A. flavus	0	1
A. fumigatus	0	1
A. glaucus	48	98
A. terreus	2	1
Penicillium sp.	46	39
Rhizopus oryzae	0	2

Table 10 (cont.).

Isolate	Percentage frequency: Dry barley	screened malt
(b)		
MFI's		
Field fungi	1.37	0.70
Storage fungi	1.02	1.56
All fungi	2.39	2.26

of the overall population among sample types revealed no clear pattern of quantitative or qualitative change in the species composition during the malting process. However, it was noted that Aspergillus clavatus was only observed in samples from dried barley, steeped grain and steep water, and Geotrichum candidum only in green, kilned and screened malt. Species detected at various stages by dilution plating but not by direct plating (Table 10) included Chrysosporium sp., Cladosporium macrocarpum, Fusarium dimerum, F. poae, Geomyces pannorum, Gibberella baccata, Monographella nivalis, Mucor hiemalis, Penicillium brevicompactum, Phoma herbarum and Verticillium lecanii, in addition to A. clavatus.

The filamentous fungi most frequently isolated from dried barley by direct plating were Alternaria alternata and species in the Aspergillus glaucus group (Raper & Fennell, 1965) and three Penicillium species, viz. P. chrysogenum, P. claviforme and P. expansum (Raper & Thom, 1949). The only other types found on >20% of kernels were Epicoccum purpurascens and Fusarium avenaceum. Although nearly all

kernels of screened malt were contaminated by A. glaucus group, the numbers contaminated by the other categories were all lower than those in the original barley, except for Absidia corymbifera and G. candidum. The latter species was not detected in barley but was isoated from one-fifth of malt kernels. The MFI values of all fungi on dried barley and screened malt were 2.39 and 2.26, respectively (Table 10). However, the lack of substantial difference in MFI's between the two sample types might be due to the high percentage frequency of contaminated screened malt by A. glaucus (98%). Nevertheless, there was a marked difference between the MFI values for dried barley (1.37) and screened malt (0.70) among the field fungi.

Yeasts

The 48 yeast isolates from dilution plates were allocated to seven genera and eight species, their characteristics matching identically of very closely the descriptions given for the species by Barnett et al. (1983) and Kreger-van Rij (1984). The species included Candida vini, C. catenulata, Rhodotorula mucilaginosa, Sporobolomyces roseus, Trichosporon beigelii, Hansenula polymorpha, Kloeckera apiculata and Debaryomyces hansenii. Details of their characteristics are given in Table 11.

The only species isolated on dilution plates of dried barley were the pink yeasts R. mucilaginosa and S. roseus, but H. polymorpha and C. vini were isolated from the first steep water. No clear pattern of development of the yeast flora during malting was apparent from the dilution plates, but the predominant species on green malt were C. catenulata and D. hansenii and on screened malt R. mucilaginosa and C.

Table 11

Characteristics of the yeasts isolated from barley corn and steep water during malt production.

Character	Presumptive identification							
	Candida catenulata	*Candida vini*	*Debaryomyces hansenii*	*Hansenula polymorpha*	*Kloeckera apiculata*	*Rhodotorula mucilaginosa*	*Sporobolomyces roseus*	*Trichosporon beigelii*
No. of isolates:	7	8	4	1	1	17	9	1
Colonial morphology:								
Red	-	-	-	-	-	-	+	-
Pink	-	-	-	-	-	+	-	-
Cream/white	+	+	+	+	+	-	-	+
Mucoid	+	+	+	-	+	+	+	+
Blastollistic	-	-	-	-	-	-	+	-
Pellicle (weak)	-	-	+	-	-	-	-	-
Pellicle (well developed)	-	-	-	-	-	-	-	+
Smooth, circular edge	-	V	-	-	+	+	+	-
Smooth, irregular edge	-	-	-	+	-	-	-	-
Rough, circular edge	-	-	+	-	-	-	-	+
Rough, irregular edge	+	-	-	-	-	-	-	+

Table 11 (cont.)

Character	Presumptive identification							
	Candida catenulata	*Candida vini*	*Debaryomyces hansenii*	*Hansenula polymorpha*	*Kloeckera apiculata*	*Rhodotorula mucilaginosa*	*Sporobolomyces roseus*	*Trichosporon beigelii*
Cell width:								
<4 µm	+	V	+	-	+	+	-	+
~4 µm	-	V	-	+	-	-	+	-
>4 µm	-	-	-	-	-	-	-	-
Cell shape:								
Spherical, 1-1.5x	-	-	+	-	-	-	-	-
Oval, 1.5-3x	-	+	-	+	+	+	+	+
Long, >3x	+	-	-	-	-	-	-	-
Pseudomycelium:								
Weak, ≤4 cells	-	-	-	-	-	-	-	-
Well developed, >4 cells	+	-	-	+	-	-	-	-
True mycelium	-	-	-	-	-	-	-	+
Fermentation of:								
D-Glucose	-	V	-	-	-	-	-	-
D-Galactose	-	-	-	-	-	-	-	-

Table 11 (cont.)

Character	Presumptive identification							
	Candida catenulata	*Candida vini*	*Debaryomyces hansenii*	*Hansenula polymorpha*	*Kloeckera apiculata*	*Rhodotorula mucilaginosa*	*Sporobolomyces roseus*	*Trichosporon beigelii*
Fermentation of:								
Sucrose	-	-	-	-	-	-	-	-
Maltose	-	-	-	-	-	-	-	-
Raffinose (R^+Mel^-)	-	-	-	-	-	-	-	-
Raffinose (R^+Mel^+)	-	-	-	-	-	-	-	-
Assimilation of:								
D-Galactose	+	-	+	+	-	-	-	+
Sucrose	+	+	+	+	-	-	-	+
Maltose	+	-	+	+	-	+	+	+
Cellobiose	-	-	+	+	+	+	+	+
Trehalose	+	-	+	+	+	+	+	+
Lactose	-	-	+	+	-	-	-	+
Melibiose	-	-	+	+	-	-	-	+
Raffinose	-	-	+	+	-	+	+	+
Starch	-	-	-	+	+	-	+	+
D-Xylose	-	V	+	-	-	+	-	+

Table 11 (cont.)

Character	Presumptive identification							
	Candida catenulata	*Candida vini*	*Debaryomyces hansenii*	*Hansenula polymorpha*	*Kloeckera apiculata*	*Rhodotorula mucilaginosa*	*Sporobolomyces roseus*	*Trichosporon beigelii*
Assimilation of:								
D-Glucose	+	+	+	+	+	+	+	+
L-Arabinose	-	-	+	+	-	+	-	-
D-ribose	-	-	-	+	-	V	-	+
Ethanol	+	+	+	+	-	+	+	+
Glycerol	+	V	+	-	-	-	-	+
Mannitol	+	V	+	+	-	+	+	+
α-methyl-glucoside	-	-	+	+	-	-	-	+
Lactic acid	+	+	+	+	-	-	-	+
Succinic acid	+	+	+	+	-	+	+	+
Citric acid	+	-	+	+	-	+	-	+
Arbutin	-	-	+	+	+	-	+	+
Lysine	+	+	+	+	+	+	+	+
KNO_3	-	-	-	+	-	-	-	-
Tolerance to:								
Vitamin-free	+	V	+	+	-	V	+	-

Table 11 (cont.)

Character	Presumptive identification							
	Candida catenulata	*Candida vini*	*Debaryomyces hansenii*	*Hansenula polymorpha*	*Kloeckera apiculata*	*Rhodotorula mucilaginosa*	*Sporobolomyces roseus*	*Trichosporon beigelii*
Tolerance to:								
20% glucose	+	+	+	+	+	+	+	+
100 ppm Actidione	+	V	+	+	+	+	+	+
Growth at:								
30° C	+	-	-	-	+	-	-	-
37° C	-	-	-	-	-	-	-	-
Capsule formation	-	-	-	-	+	+	+	+
Polar budding	-	-	-	-	+	-	-	-
Binary fission	-	-	-	-	-	-	-	+
Good anaerobiosis	+	V	+	-	-	-	-	-
Spore formation	-	-	-	-	-	-	-	-

+ = 75 - 100%; V = 21 - 74%; and - = <20% positive responses for the characteristics.

catenulata. Moreover, R. mucilaginosa was detected on all sample types and S. roseus on all samples except first steep water and green malt. Candida vini was isolated from all samples except dried barley and first steeped grain.

Bore-hole water

Examination of the bore-hole water revealed that virtually no yeasts nor filamentous fungi were detected. Total bacteria were approximately 1 cell ml^{-1} water, and comprised cream-coloured aeromonads, lactobacilli and pseudomonads. No Escherichia coli was detected. Detailed characterization of the isolates was not carried out. Hence, identification of the micro-organisms did not go beyond testing for the production of catalase, oxidase, Gram reaction and colonial morphology (see Appendix 3).

Physical Characteristics of Barley and Malt

Some physical characteristics of dried barley and the screened malt are shown in Table 12. The moisture content of dried barley and screened malt were 10.4% and 3.9%, respectively. The values for water sensitivity, germination energy and capacity (H_2O_2) for barley were 48%, 98% (4 ml H_2O, 18° C, 72 h) and 75% (H_2O_2), respectively.

Microbial Status of Barley During the Preparation of Control and Inoculated Malts in Malting Run 1

Control malts:

The filamentous fungi used in the inoculation of the barley for malting were detected on dilution and direct-plating plates (Tables

Table 12

Some characteristics of the dry barley grain and the malt.

Test sample	Moisture content	Water sensitivity	Germination capacity	Germination energy
Dry barley	10.4%	48%	75% (H_2O_2)	98% (4 ml H_2O, 18° C, 72 h)
Screened malt	3.9%			

13a, 14a, and Appendices 4 and 5. Fusarium nivale was only detected on barley after the second steep and in first steep water, with a MFI of 0.29 and viable count of 0.1 x 10^2 units ml^{-1} (1.7%), respectively. In contrast, Geotrichum candidum was observed on green, kilned and screened malts in dilution plating. The index for G. candidum rose gradually from 0.16 (on first steeped barley) through 0.22 (on second steeped barley), and peaked with 0.86 (on green malt), after which it declined with kilning (0.16) and screening (0.34) (Table 14a). The failure to record any G. candidum on dried barley, and first and second steeped grains may have been because the population levels were below the level of detection by dilution plating. Indeed, this fungus appeared on these sample types in direct plating technique. Although the index for G. candidum at kilning was low (0.16), the total MFI at this stage was considerably high (1.74). This high level of contamination was mainly due to increased number of Mucor spp. with a 54% contamination level. The screened malt showed an increase in the index when compared with kilned malt (Table 14a), indicating that the

Table 13a

Average viable counts of 'applied' micro-organisms in steepwater and on barley throughout the preparation of control malts in malting Run 1.

'Applied' Micro-organisms	Malt	Dried barley VU K^{-1}	Barley first steep VU K^{-1}	Barley second steep VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU K^{-1}	Second steep water VU K^{-1}
F. nivale	CFm5	-	-	-	-	-	-	$0.1x10^2$	-
		$(2.2x10^3)$	$(1.3x10^2)$	$(1.0x10^2)$	$(1.8x10^4)$	$(9.4x10^2)$	$(1.7x10^2)$	$(5.8x10^2)$	$(6.4x10^2)$
G. candidum	CFm7	-	-	-	$1.3x10^4$	$1.0x10^2$	$2.7x10^2$	-	-
		$(1.1x10^3)$	$(1.0x10^2)$	$(1.4x10^3)$	$1.3x10^4$	$(3.4x10^2)$	$(5.1x10^2)$	$(1.0x10^3)$	$(1.4x10^3)$

Table 13b

Average viable counts of 'applied' micro-organisms in steep water and on barley throughout the preparation of inoculated malts in malting Run I.

'Applied' Micro-organisms	Malt	Inoculum VU K^{-1}	Barley first steep VU K^{-1}	Barley second steep VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU K^{-1}	Second steep water VU K^{-1}
F. nivale	TFm5	$1.0x10^4$	80	$1.0x10^2$	-	-	-	$0.9x10^2$	-
			$(1.3x10^3)$	$(5.5x10^2)$	$(1.3x10^6)$	$(5.3x10^4)$	$(2.3x10^4)$	$(2.0x10^4)$	$(2.2x10^3)$
G. candidum	TFm7	$7.8x10^5$	$2.1x10^5$	$9.6x10^3$	$2.3x10^6$	$2.4x10^5$	$5.8x10^5$	$4.6x10^5$	$7.5x10^6$
			$(2.1x10^5)$	$(9.6x10^3)$	$(2.3x10^3)$	$(2.4x10^5)$	$(5.8x10^5)$	$(4.6x10^5)$	$(7.5x10^6)$

- denotes not detected; figures in parentheses denote total mould populations.

VU K^{-1} denotes viable units per kernel.

Table 14a

Mould frequency index of 'applied' micro-organisms during the preparation of control malts in malting Run I.

'Applied' Micro-organisms	Malt	Dried barley	Barley after first steep	Barley after second steep	Green malt	Kilned malt	Screened malt
F. nivale	CFm5	-	-	0.29	-	-	-
		1.98	1.49	(1.76)	(1.78)	(2.13)	(1.93)
G. candidum	CFm7	-	0.16	0.22	0.86	0.16	0.34
		1.98	(1.96)	(1.60)	(2.48)	(1.74)	(2.08)

Table 14b

Mould frequency index of 'applied' micro-organisms during the preparation of inoculated malts in malting Run I.

'Applied' Micro-organisms	Malt	Inoculum VU ml^{-1}	Barley after first steep	Barley after second steep	Green malt	Kilned malt	Screened malt
F. nivale	TFm5	$1.0x10^4$	0.12	-	-	-	-
			(1.15)	(1.31)	(2.00)	(1.90)	(1.73)
G. candidum	TFm7	$7.8x10^5$	1.00	1.00	1.00	1.00	0.98
			(1.92)	(2.18)	(2.26)	(2.08)	(2.18)

- Not detected; Figures in parentheses denote total MFI

VU ml^{-1} denotes viable units per ml.

screening process did not remove much of the contaminants and/or perhaps multiplication of the existing micro-organisms ensued.

There was a diverse microbial population in the samples other than those mentioned above (Fig. 7a; Appendices 4 and 5), including aerobic heterotrophic bacteria, lactic acid bacteria, yeasts and filamentous fungi. The total bacterial population increased to 1.5×10^8 units kernel^{-1}, with lactic acid bacteria recorded at 6.5×10 units kernel^{-1}, yeasts at 3.3×10^5 units kernel^{-1} and moulds at 1.8×10^4 units kernel^{-1} on green malt (Fig. 7a; Appendix 4a, b).

Inoculated malts:

The mould frequency indices and the viable count of micro-organisms applied during malting are given in Tables 14b and 13b, respectively. Despite the high initial inoculum (1.0×10^4 units ml^{-1}) at the first steep stage, the growth of F. nivale had declined during malting, so much that no viable cells were detected as from the green malt, either in dilution or direct plating. The 80 units kernel^{-1} detected on first steeped grain only formed approximately 6% of the total mould population, although the first steep water contained a negligible proportion (0.5%). However, the steeped-out barley harboured 1.0×10^2 units kernel^{-1}, i.e. 18% of the total mould detected in the sample. Compared with the total MFI (1.15), F. nivale scored only 0.12. Whether the poor growth was ecological (which presumably may likely be the reason) or otherwise, one interesting observation was that the inoculated samples showed as poor growth of F. nivale as the control. In contrast, G. candidum showed a profuse growth throughout the malting process (Tables 13b and 14b; Appendices 6 and 7). The viable counts of G. candidum applied in first steep water

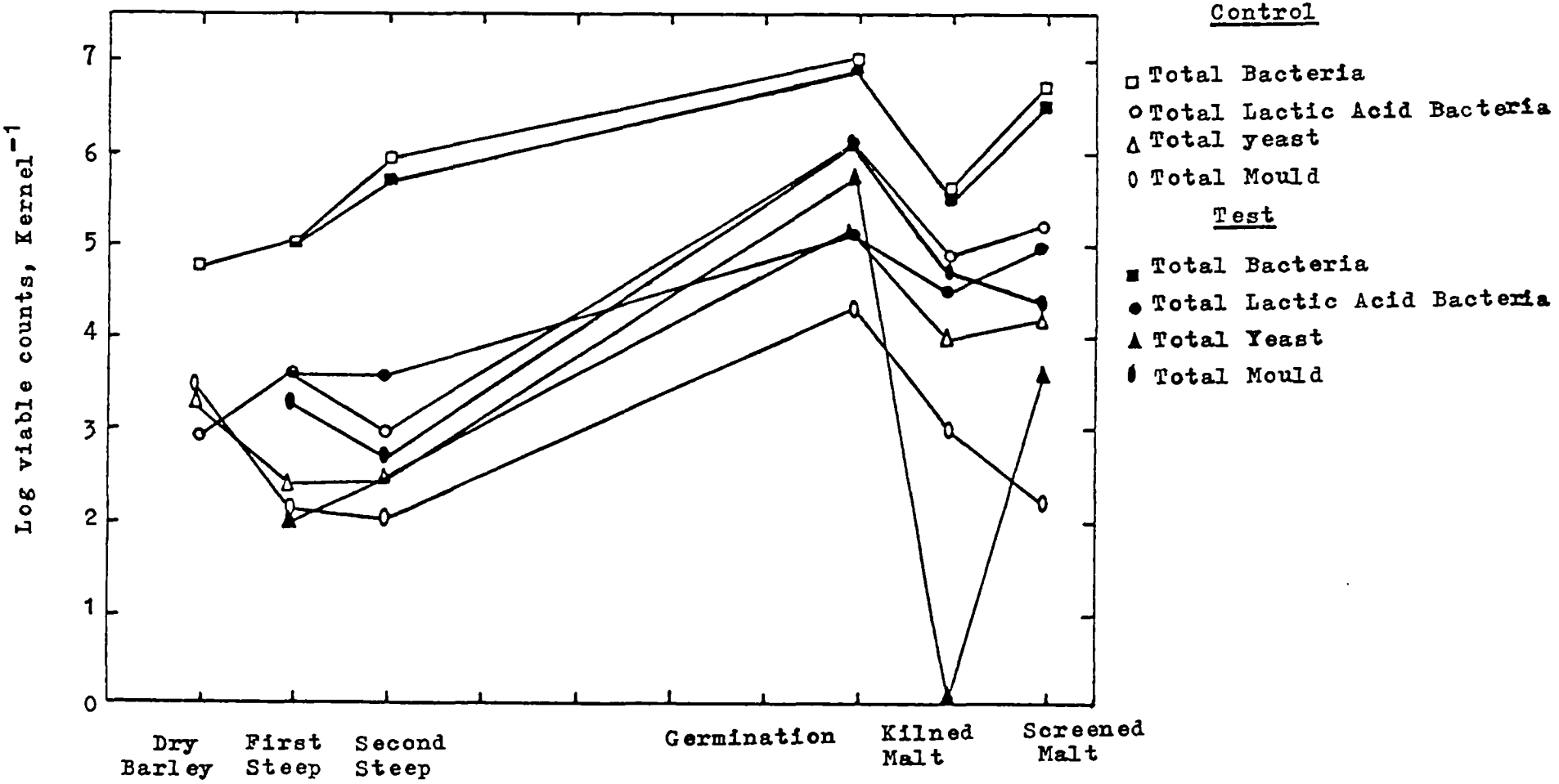


Figure 7a. Total viable counts of micro-organisms(by dilution plating) during preparation of Fusarium nivale inoculated malts in malting Run 1.

ranged from 8.3 x 10^3 units $kernel^{-1}$ (second steeped barley) to 7.5 x 10^6 units ml^{-1} (second steep water) throughout the malting process. Invariably, these counts formed approximately 100% each of the total moulds recovered in each sample type, indicating the dominant potential. Similar dominance was observed by direct plating. Virtually all kernels were contaminated with G. candidum (Table 14b). However, the presence of Aspergillus glaucus at an equally high frequency was noted (Appendix 7b). These high levels contributed to high MFI. The kilning process did not seem to effect a reduction in the number of kernels contaminated by G. candidum. However, there was an apparent reduction from 2.3 x 10^6 unit $kernel^{-1}$ (green malt) to 2.4 x 10^5 units $kernel^{-1}$ (kilned malt) recorded in dilution plating. Comparing the inoculated with control malts, it was observed that G. candidum was detected on the first and second steeped inoculated grains and in first and second steep water. In contrast, no growth was detected on similar control samples. In all cases, the viable counts on the inoculated malts were higher than those in control malts. A similar situation occurred with F. nivale. Here again, the microorganisms in the inoculated malt samples consisted of aerobic heterotrophic and lactic acid bacteria, moulds and yeasts (Fig.7a; Appendices 6 and 7). It was noted that Penicillium sp. dominated at all stages of malting (Appendices 6 and 7), despite the very low viable counts on the dried barley and on the control malts.

Microbial Status of Barley During the Preparation of Inoculated and Control Malts in Malting Run II

Control malts

The presence or absence of the inoculated micro-organisms in the control malts was noted (Tables 15a and 16a; Appendices 8a and 9a). Although small populations of F. nivale were detected in malting Run No. I, none was recovered from the control malts throughout the malting process in Run II, in either direct or dilution plating. However, as in the previous Run, there was some growth of other moulds throughout the different stages of malting with populations ranging from 1.0×10^2 (kilned malt) to 6.9×10^2 units kernel^{-1} (first steeped barley). Moreover, total bacteria (6.1×10^3, dried barley - 9.1×10^7units kernel^{-1}, green malt), lactic acid bacteria (40, dried barley - 8.0×10^4 units kernel^{-1}, green malt), and yeasts (40, dried barley - 1.2×10^6 units kernel^{-1}, green malt) were also recovered from all sample types during malting (Fig. 7b, Appendices 8a and 9a). Aspergillus glaucus predominated on all sample types with 98 - 100% frequency of contamination of kernels. Of course, the steep water contained large numbers of all different types of micro-organisms.

G. candidum, as in Run No. I, was detected on barley, at all stages of malting (including dried barley) (Table 16a) in direct plating. However, the detection was only confined to green, kilned and screened malt in dilution plating (Table 15a). In both cases, green malt harboured the highest number of the organisms, i.e. up to 1.1×10^4 units kernel^{-1} (dilution plating) and 100% kernel contamination (direct plating). The total viable counts of micro-organisms detected during malting (Fig. 7b; Appendices 8b and 9b) ranged from 1.4×10^5 units kernel^{-1} (first steeped barley) to 7.0×10^6 (green malt) for

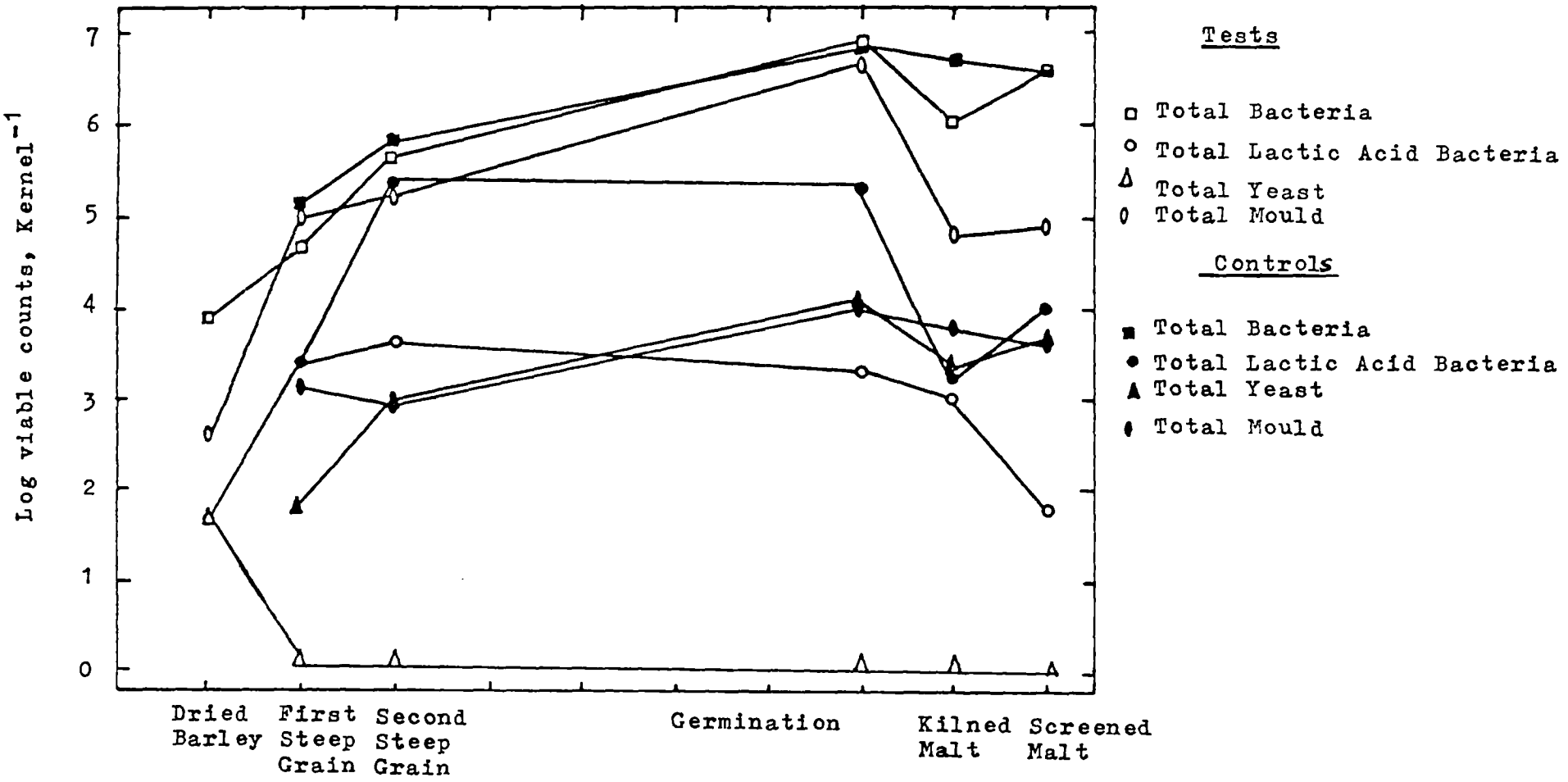


Figure 7b. Total viable counts of micro-organisms (by dilution plating) during preparation of control and Geotrichum candidum inoculated malts in malting Run 2.

Table 15a

Average viable counts of 'applied' micro-organisms in steep-water and on barley throughout the preparation of control malts in malting Run II.

'Applied' Micro-organism	Malt	Dried barley VU K^{-1}	Barley first steep VU K^{-1}	Barley second steep VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
F. nivale	CFm5	-	-	-	-	-	-	-	-
		($2.9x10^2$)	($6.9x10^2$)	($4.5x10^2$)	($2.6x10^2$)	($1.0x10^2$)	($2.2x10^2$)	($2.6x10^3$)	($3.0x10^2$)
G. candidum	CFm7	-	-	-	$1.1x10^4$	$4.7x10^3$	$1.7x10^3$	-	-
		($2.9x10^2$	($1.2x10^3$)	($7.7x10^2$)	($1.1x10^4$)	($6.4x10^3$)	($3.7x10^3$)	($5.0x10^2$)	($6.7x10^2$)

Table 15b

Average viable counts of 'applied' micro-organisms in steep-water and on barley throughout the preparation of inoculated malts in malting Run II.

'Applied' Micro-organism	Malt	Inoculum VU K^{-1}	Barley first steep VU K^{-1}	Barley second steep VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
F. nivale	TFm5	$1.4x10^3$	$1.5x10^3$	$2.0x10^2$	$1.0x10^3$	-	-	$3.0x10^2$	-
			($1.8x10^3$)	($6.0x10^2$)	($1.4x10^3$)	(50)	-	($1.1x10^3$)	($2.7x10^2$)
G. candidum	TFm7	$1.2x10^6$	$1.0x10^5$	$1.5x10^5$	$4.9x10^6$	$5.7x10^4$	$7.9x10^4$	$2.4x10^7$	$2.9x10^7$
			($1.0x10^5$)	($1.5x10^5$)	($4.9x10^6$)	($5.7x10^4$)	($7.9x10^4$)	($2.4x10^7$)	($2.9x10^7$)

- Not detected; Figures in parentheses denote total mould populations.
VU K^{-1} denotes viable units per kernel.

Table 16a

Mould frequency index (MFI) of 'applied' micro-organisms during the preparation of control malts in malting Run II.

'Applied' Micro-organism	Malt	Dried barley	Barley after first steep	Barley after second steep	Green malt	Kilned malt	Screened malt
F. nivale	CFm5	-	-	-	-	-	-
		(1.79)	(1.74)	(1.36)	(1.90)	(1.56)	(2.00)
G. candidum	CFm7	0.4	0.60	0.48	1.00	0.92	0.12
		(1.79)	(1.94)	(1.72)	(2.22)	(1.94)	(1.88)

Table 16b

Mould frequency index (MFI) of 'applied' micro-organisms during the preparation of inoculated malts in malting Run II.

'Applied' Micro-organism	Malt	Inoculum VU ml^{-1}	Barley after first steep	Barley after second steep	Green malt	Kilned malt	Screened malt
F. nivale	TFm5	$1.4x10^3$	0.26	0.16	-	-	0.02
			(1.64)	(1.34)	(1.62)	(1.54)	(1.74)
G. candidum	TFm7	$1.2x10^6$	1.00	1.00	1.00	0.60	1.00
			(2.12)	(2.10)	(2.00)	(2.02)	(2.06)

- Not detected; Figures in parentheses denote total MFI.
VU K^{-1} denotes viable units per kernel.

bacteria; 2.0 x 10^3 units kernel^{-1} (first steeped barley) to 2.6 x 10^5 (steeped out barley) for lactic acid bacteria; 70 units kernel^{-1} (first steeped barley) to 1.3 x 10^4 (green malt) for yeasts; and 7.7 x 10^2 units kernel^{-1} (steeped out barley) to 1.1 x 10^4 (green malt) for moulds. Again, the high levels of Asp. glaucus contamination of kernels was noted, ranging from 72 to 100% in all sample types.

Inoculated malt:

The viable units of Fusarium nivale were detected in more sample types (Tables 15b and 16b), than in Run I. In dilution plating, these included first steeped grain (1.5 x 10^3 units kernel^{-1}; 83% of the total moulds in the sample); steeped out barley (2.0 x 10^2 units kernel^{-1}; 33% of total moulds in the sample); green malt (1.0 x 10^3 units kernel^{-1}, 71% of total moulds in the sample); and first steep water (3.0 x 10^2 units ml^{-1}, 27% of total moulds in the sample). Indices of 0.26, 0.16 and 0.02 for first and second steeped barley and screened malt, respectively were recorded. This was the first time F. nivale was detected on screened malt in the two runs and at very low frequency (2%). Since F. nivale was not detected in the control malts, it invariably meant proliferation of these micro-organisms in the samples. Nevertheless, kilning had reduced the population to a level below detecion in the case of kilned and, perhaps, screened malt. However, the mould was not detected in green malt by direct plating but by dilution plates (Tables 15b, 16b).

There were other diverse micro-organisms in the various samples throughout malting (Appendices 10a and 11a). These included aerobic heterotrophic bacteria, lactic acid bacteria, yeasts and moulds. Thus,

the total viable counts of G. candidum applied in the first steep water during malting have been included in Table 15b. Populations ranged from 5.7 x 10^4 units kernel^{-1} (kilned malt) to 4.9 x 10^6 units kernel^{-1} (green malt). All sample types contained the applied micro-organism. The populations in the second steep water did not change substantially from the level in the first steep water. On all sample types, the viable counts of the inoculated mould (G. candidum) were higher than those of the corresponding control malt. However, the population of G. candidum on screened malt in malting Run II was lower than that in malting Run I and these were in the order of 7.9 x 10^4 and 5.8 x 10^5 units kernel^{-1}, respectively, but higher on green malt (4.9 x 10^6 and 2.3 x 10^6 units kernel^{-1}, respectively). G. candidum dominated the mould population (100%) both in malting Runs I and II in dilution plating. Unlike other micro-organisms, yeasts were not detected at all in any of the samples (Appendix 10b).

Physical Analysis

Moisture content during malting:

During malting there were changes in moisture content of barley from the first steeped barley to screened malt (Table 17a, b). In Run I with F. nivale inoculated malt, the moisture contents (MC) of the first and second steeped grain in the inoculated grain were appreciably higher than those for corresponding samples in control grains. The difference being 3 and 2.2% in first and second steeped barley, respectively. There was no appreciable difference in MC of other sample types. With G. candidum inoculatd malts, the MC of barley were lower in all sample types than those of the control malts (Table 17a).

Table 17a

Moisture content (% wet weight) of kernels during the preparation of controls and inoculated malts in malting Run I.

Malt	Moisture Content (% wet weight)				
	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
Controls:					
CFm5	36.9	37.9	46.0	3.5	3.6
CFm7	36.9	42.1	44.5	4.2	4.8
Tests:					
TFm5	39.4	40.1	45.7	3.4	3.7
TFm7	33.8	41.7	42.7	3.6	4.1

NB. %MC (wet weight) of dried barley equalled 14.5.

Table 17b

Moisture content (% wet weight) of kernels during the preparation of controls and inoculated malts in malting Run II.

Malt	Moisture Content (% wet weight)				
	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
Controls:					
$CFm5_2$	37.3	37.3	44.3	3.9	4.1
$CFm7_2$	37.7	41.7	44.8	3.7	6.6
Tests:					
$TFm5_2$	37.4	40.4	46.9	3.5	3.7
$TFm7_2$	37.0	41.6	43.2	3.6	5.9

NB. % MC (wet weight) of dried barley equalled 14.6. TFm5 indicates malt inoculated with Fusarium nivale; TFm7 indicates malt inoculated with Geotrichum candidum.

In malting Run II, with F. nivale inoculated malts, the MC of steeped out barley and green malt were noticeably higher than those of the corresponding control samples by 3.1 and 2.6%, respectively. Whereas the first steeped grain showed virtually no difference in MC between the control and inoculated malts, the MC of kilned and screened malts were higher in the control than in inoculated malt (Table 17b). This may suggest an absence of involvement of micro-organisms, which might have been reduced or killed during kilning.

Barley inoculated with G. candidum showed a similar pattern of MC for each sample type to the corresponding MC in Run I. Furthermore, although lower in inoculated malts than in controls, the MC differences were not appreciable (~0.1 - 0.7% MC) (Table 17b).

Malt Recovery and Root, Steep and Respiration Losses

The malt recovery and root, steep and respiration losses have been detailed in Table 18. In malting Run I (Table 18a), the percentage recovery of inoculated malts was slightly lower than the values for their corresponding controls. The root loss recorded in malt inoculated with F. nivale was higher than in the control by 1.5%. Conversely, malt inoculated with G. candidum scored lower root loss (1.3%) than the control (3.0%). The combined steep and respiration loss in F. nivale inoculated malt showed a lower value (7.8%) than the control (8.1%). In contrast, there was a higher steep and respiration loss in G. candidum inoculated malt than the values for the control. The difference was 3%.

In malting Run II (Table 18b), the percentage recovery of F. nivale inoculated malt was higher by 1.9% than the value for control

malt. Similarly in G. candidum inoculated and control malt, the values were very close, ie. 87.8 and 88.0%, respectively. However, the range of percentage malt recovery in both Runs was close. All values fell between 86.6 - 89.4% (controls) and 87.1 - 88.0% (inoculated malts).

Root loss followed the same pattern as in Run I, but with a

Table 18a

Malt recovery and root, steep and respiration losses of controls and inoculated malts in malting Run I.

Malt	Recovery (%)	Root loss (%)	Steep + Respiration loss (%)
Controls:			
CFm5	88.9	3.0	8.1
CFm7	89.4	2.9	6.2
Tests:			
TFm5	87.1	4.5	7.8
TFm7	88.0	1.3	9.0

Table 18b

Malt recovery and root, steep and respiration losses of controls and inoculated malts in malting Run II.

Malt	Recovery (%)	Root loss (%)	Steep + Respiration loss (%)
Controls:			
CFm5	86.6	3.7	8.5
CFm7	88.0	4.7	7.1
Tests:			
TFm5	87.5	4.2	8.2
TFm7	87.8	3.0	9.1

TFm5 indicates malt inoculated with F. nivale; TFm7 indicates malt inoculated with G. candidum.

narrower difference between controls and inoculated malt. In combined steep and respiration loss, the pattern again was similar to that described in Run I, but with smaller differences between the controls and the inoculated malts.

Length of Acrospire at 5 day Germination of Control and Inoculated Barleys in Malting Runs I and II

The growth indices of both controls and inoculated malts were very close in the two malting Runs (Table 19).

Chemical Analyses

Chemical characteristics of control and inoculated malts

In determining the significance of any difference between controls and inoculated malts, Student's 't' test (Appendix 12) was used at 95% confidence limits and degree of freedom n-1 = 2.

Malting Run I:

The characteristics of the chemical analyses for malting Run I have been included in Table 20a. The diastatic power values of the control malts were all higher than the corresponding data for inoculated malts. In particular, F. nivale and G. candidum inoculated malts, were lower by 6 and 7° L, respectively, than the values for the controls and these were significantly different. The levels of reducing sugars present in the malt samples before DP determination were noted. These were: 68 ppm each for control and F. nivale inoculated malts; and 67 ppm and 68 ppm for control and G. candidum inoculated malts, respectively.

The commercial malt recorded and the experimental malts passed

Table 19

Growth index of acrospire at 5 d germination of controls and inoculated barleys in malting Runs I and II.

Length of acrospire	Growth Index			
	Control	TFm5	Control	TFm7
Run I				
Dead	0	0	0	0
0 - 1/4	0.25	0.25	0	0
1/4 - 1/2	1.00	2.00	0	0
1/2 - 3/4	8.25	9.00	3.75	3.00
3/4 - 1	77.00	74.00	88.00	89.00
Overgrown	10.50	13.50	7.50	7.50
Run II				
Dead	0	0	0	0
0 - 1/4	0	0	0	0
1/4 - 1/2	0	0	6.0	6.0
1/2 - 3/4	0	0	24.0	25.5
3/4 - 1	98.0	84.0	52.0	52.0
Overgrown	1.5	24.0	4.5	3.0

TFm5 denotes barley inoculated with F. nivale; TFm7 denotes barley inoculated with G. candidum.

Table 20a

Chemical characteristics of commercial, control and inoculated malts in malting Run I.

Characteristic	MALTS				
	Commercial malt from Pencaitland	Control	TFm5	Control	TFm7
Diastatic power (°L)	102	88.0±0.33	82*	93	86*
Colour (°EBC unit)	2.6	2.3±0.10	2.6	2.8	2.2*
Total nitrogen (N) (% dry matter)	1.74	1.5	1.52	1.49	1.57
Total soluble N (% dry matter)	0.82	0.98	0.89	0.81	0.78
Total soluble N / total N %	47	65	59	54	50
α-amino N (mg^{-1})	137.5	200.1±0.9	188.6*	176.0±0.7	174.1
% friability	74.9	74.8±0.03	72.3	72.5	69.0
Hot water extract fine grind (°l kg^{-1})	305	324.0	324.9	324.8	321.2
Hot water extract coarse grind (°l kg^{-1})	301.5	321.6±0.33	315.0*	321.0±0.33	311.0*
Fine-coarse difference	3.5	2.4	9.9	3.8	10.2
Cold water extract (%)	17.3	22.8±0.2	21.8	23.3	20.2
% fermentability	75.2	73.9±0.3	76.7*	76.1±0.52	75.7
Fermentable extract (°l/kg)	220.9	224.58±0.9	233.09*	231.27	230.05
% ethanol (fermented wort)	2.43	2.50±0.03	2.72	2.63±0.03	2.61

*95% confidence limit; TFm5 denotes malt inoculated with Fusarium nivale, TFm7 denotes malt inoculated with Geotrichum candidum.

through diferent environmental malting procedures, hence there was no basis for comparison. Wort colour of F. nivale inoculated malts was higher by 0.3 °EBC unit than the controls, but not significantly different. In contrast, G. candidum inoculated malt recorded lower units than the control. The difference was 0.6 °EBC unit and significant.

Without adjusting for the contributions made by the applied fungal biomass, the nitrogenous compounds and the extracts were compared as observed. Both total malt nitrogen (TN) and total soluble nitrogen (TSN) values of the inoculated malts did not differ significantly from those of their corresponding controls. However, surprisingly, the values for the inoculated malts were all lower than those of the corresponding controls. The difference fell between 0.02 - 0.08% (TN), and between 0.03 - 0.08% (TSN). However, the values for the TSN/TN ratio, which is regarded as the index of protein modification of malt, were greater than the limits (33 - 37%) of well modified malts (Briggs et al., 1981) for both the controls and the inoculated malts. Levels of free α-amino nitrogen in worts prepared from control malts were higher in all cases than those of inoculated malts. However, significantly different values were only recorded from F. nivale inoculated malt wort.

The percentage friability of inoculated malts were all lower, although not significantly, than the values for the controls. The values ranged from 69.0 to 74.9%. Hot water extracts (HWE, fine grind) were not significantly different in both the controls and the inoculated malts. In contrast, the hot water extracts (HWE, coarse grind) of inoculated malts were substantially lower than those for controls, and also significantly different. These low coarse grind HWE

recorded for the inoculated malts contributed to the high coarse-fine difference. F. nivale inoculated malt differed by 7.5 °l kg^{-1} and G. candidum inoculated malt by 6.4 °l kg^{-1} from the controls. Cold water extracts (CWE) of the inoculated malts were noted to be slightly lower than those of controls, but were not significantly different. Both the fermentable extracts and percentage fermentability for G. candidum inoculated malts differed marginally from the controls and were not significantly different. Conversely, these two parameters were greater than, and significantly different in the F. nivale inoculted malts than in their corresponding controls. The differences were 2.8% (% fermentability) and 8.5 °l kg^{-1} (fermentable extract).

The amount of ethanol (%) obtained from fermentation of wort derived from F. nivale inoculated malt was greater than the value for control but not significantly so. The yield for G. candidum inoculated malt was marginally lower than the value for the control but not significantly lower.

The values for the commerically prepared malt were included as checks but not for comparison because the two categories of samples passed through different malting procedures, i.e. commercial and laboratory maltings.

Malting Run II:

The chemical characteristics for malting Run II are given in Table 20b.

The diastatic power (DP) of malts in malting Run II were the same for control and F. nivale inoculated malts (94 °L), but significantly different for G. candidum inoculated malt (7 °L) and control malt.

Table 20b

Chemical characteristics of commercial, control and inoculated malts in malting Run II.

Characteristic	MALTS				
	Commercial malt from Pencaitland	Control	TFm5	Control	TFm7
Diastatic power (°L)	102	94	94	93	86*
Colour (°EBC unit)	2.6	2.2	2.5	2.7±0.01	2.5
Total nitrogen (N) (% dry matter)	1.74	1.51	1.51	1.55±0.01	1.57
Total soluble N (% dry matter)	0.82	0.81	0.76	0.89	0.80
Total soluble N / total N %	47	54	50	57	51
α-amino N (mg^{-1})	137.5	193.4±0.17	190.5*	204.9±10	197.2*
% friability	74.9	74.8±0.03	73.2	72.0±0.03	69.0
Hot water extract fine grind (°l kg^{-1})	305	326.0±0.3	323	325±0.30	321*
Hot water extract coarse grind (°l kg^{-1})	301.5	322.8±0.3	320	322.8±0.30	318.7*
Fine-coarse difference	3.5	3.2	3.0	2.2	2.3
Cold water extract (%)	17.3	22.1±0.1	23.0	21.6±0.20	21.8
% fermentability	75.2	77.3±1	76.9	76.2±0.06	76.1
Fermentable extract (°l/kg)	220.9	233.9±1	233.7	231.4±0.30	231.3
% ethanol (fermented wort)	2.43	2.67±0.04	2.77	2.64±0.04	2.60

*95% confidence limit; TFm5 denotes malt inoculated with Fusarium nivale, TFm7 denotes malt inoculated with Geotrichum candidum.

The initial levels of reducing sugars in the malt infusion were as follows: F. nivale inoculated malt and its control contained 68 ppm and 63 ppm, respectively, and G. candidum inoculated malt revealed 70 ppm and 68 ppm (fructose).

There was no significant difference in the values obtained for colour of the worts prepared from both control and inoculated malts. These ranged from 2.2 to 2.7 °EBC units.

Similarly to Run I, no significant differences existed between the corresponding controls and inoculated malts in the values of TN and TSN. The values for α-amino nitrogen followed the same pattern, but were significantly lower than the values for control malts, up to 2.9% (F. nivale inoculated malts) and 7.7% (G. candidum inoculated malts).

The percentage friability of the malts was not significantly different amongst sample types and the values compared very closely with the corresponding results in malting Run I.

The values for HWE (fine grind) were lower in the inoculated malt wort than in the controls. However, only the extract from the G. candidum inoculated malt showed a significant difference. The HWE (coarse grind) followed the same pattern, although no appreciable difference was observed in the corresponding fine-coarse values. The differences between the values for G. candidum inoculated malt and the control were 0.1%, and for F. nivale inoculated malt 0.2%. These were different from the high significant values obtained in malting Run I.

As in malting Run I, there were no significant differences in the values for CWE among the sample types. Fermentable extracts and percentage fermentabilities in all sample types did not show any appreciable differences between the corresponding controls and inoculated malt fermentation extracts. Again, the values compared

closely to those of malting Run I.

As with malting Run I, the values for the commercial malts were used only as checks and not for comparison, for the same reason as given in the previous section (malting Run I).

Effects of Extracellular Enzymes on Some Carbohydrates Associated with Barley or Malt

Micro-organisms showing xylanolytic, amylolytic and glucanase activities on plates at 25° C

Of the 32 bacterial cultures screened for the production of xylanolytic, amylolytic and glucanase activities, only Flavobacterium esteroaromaticum (3% of the total number of bacteria) was observed to produce diffusible xylanase; 12 strains exhibited amylolytic activity (32% of the total number of bacteria), and nine cultures gave positive responses to glucanase activity (28% of the total number of bacteria (Table 21a).

The reactions of 37 filamentous fungi and eight yeast species towards xylan, soluble starch and barley-β-glucan have been included in Table 21b. For xylanolytic activity, 20 filamentous fungi (54% of the total number of moulds) but no yeasts, showed positive responses; for amylolytic activity, 24 cultures of filamentous fungi (65% of the total number of moulds) gave positive reactions. Only 25% of the yeasts showed weakly positive reactions. For glucanase activity, 15 filamentous fungi (41% of the total number of moulds) gave positive reactions, whereas the yeasts again reacted weakly (25% were positive). Overall, it was indicated that more cultures of filamentous fungi expressed the extracellular enzyme activity on plates than either

Table 21a

Bacterial flora showing xylanolytic, amylolytic and glucanase activities on plates at 25° C

Bacteria	Xylanolytic activity	Amylolytic activity	Glucanase activity
Alcaligenes sp.	-	-	-
Chromobacterium sp.	-	-	-
Yellow chromogens	-	-	-
Bacillus sp.	-	-	d
Bacillus sp.	-	-	d
Bacillus sp.	-	+	d
Corynebacterium sp.	-	+	-
Flavobacterium esteroaromaticum	+	+	d
Clavibacter iranicum	-	+	d
Microbacterium imperiale	-	+	d
M. lacticum	-	-	d
Arthrobacter globiformis	-	+	d
Aureobacterium flavescens	-	+	-
Brevibacterium linens	-	+	-
Aur. flavescens	-	-	-
Corynebacterium sp.	-	+	-
Cla. michiganese	-	d	-
Oerskovia xanthineolytica	-	-	-
Oer. turbata	-	+	-
Serratia plymuthica	-	-	-
S. rubidaea	-	-	-
Erwinia herbicola	-	-	-

Table 21a (cont.)

Bacteria	Xylanolytic activity	Amylolytic activity	Glucanase activity
Pseudomonas fluorescens	-	-	-
Ps. putida	-	-	-
Ps. putida	-	-	-
Lactobacillus sp.	-	-	-
Lactobacillus sp.	-	-	-
Lactobacillus sp.	-	-	-
L. alimentarius	-	-	-
L. plantarum	-	-	-
L. acidophilus	-	-	-
Bacillus sp. W12	d	+	+

+ = positive response; - = negative response; d = weak (doubtful) response.

Table 21b

Mycoflora showing xylanolytic, amylolytic and glucanase activities on plates at 25° C.

Moulds	Xylanolytic activity	Amylolytic activity	Glucanase activity
Absidia corymbifera	-	-	d
Alternaria alternata	-	-	-
Aspergillus candidus	-	d	-
A. clavatus	+	+	+
A. flavus	+	+	+
A. fumigatus	+	+	-
A. glaucus	-	-	-
A. terreus	+	+	+
Aureobasidium pullulans	+	+	-
Botrytis cinerea	+	+	-
Chrysosporium sp.	-	-	-
Cladosporium cladosporioides	-	+	+
C. macrocarpum	-	-	+
Cochliobolus sativus	+	d	-
Epicoccum purpurascens	+	+	+
Fusarium avenaceum	d	d	+
F. dimerum	-	-	+
F. lateritium	-	-	+
F. nivale	+	+	+
F. poae	-	d	-
Geotrichum candidum	-	-	-
Mucor hiemalis	-	-	+

Table 21b (cont.)

Moulds	Xylanolytic activity	Amylolytic activity	Glucanase activity
Penicillium sp.	+	+	+
Penicillium sp.	+	+	-
P. brevi-compactum	+	+	-
P. chrysogenum	+	+	+
P. claviforme	+	+	-
P. expansum	+	+	+
Phoma herbarum	+	+	-
Rhizopus oryzae	-	-	-
Verticillium lecanii	-	-	-
Unidentified moulds:			
Fm22	-	d	-
Fm23	+	d	-
Fm24	-	-	-
DB14	-	-	-
DB15	+	+	-
DB16	+	+	-
Yeasts:			
Candida catenulata	-	-	-
C. vini	-	-	-
Debaryomyces hansenii	-	-	-
Hansenula polymorpha	-	d	d
Kloeckera apiculata	-	-	-
Rhodotorula mucilaginosa	-	-	d
Sporobolomyces roseus	-	d	-

Table 21b (cont.)

Moulds	Xylanolytic activity	Amylolytic activity	Glucanase activity
Yeasts:			
Trichosporon beigelii	-	-	-

+ = positive response; - = negative response; d = weak (doubtful) response.

bacterial or yeast cultures.

Effect of days of incubation on microbial β-glucanase production in liquid media at 25° C

The amount of β-glucanase produced was determined, based on the hydrolysis of β-glucan to produce glucose. Hence, measurement of glucose concentration was the index of measurement. The effect of numbers of days of incubation on microbial β-glucanase production is shown in Table 22 and Fig. 8. The second day (48 h) of incubation seemed to be the best period to grow the bacterial cultures in order to produce maximum levels of β-glucanase. This period was noted for all the bacteria tested. Bacillus sp. (W12) gave the highest activity (10.3 µg ml^{-1} glucose released 0.5 ml^{-1} enzyme extract). However, this organism was not isolated from barley. The strain of Bacillus (A6) isolated from the malting barley gave 9.3 ug ml^{-1} glucose 0.5 ml^{-1} enzyme extract). The least glucanase production came from Arthrobacter globiformis (1.2 µg ml^{-1} glucose 0.5 ml^{-1} enzyme extract).

Fig. 8 and Table 22 show the effect of incubation period on fungal production of β-glucanase. Five days appeared to be the best

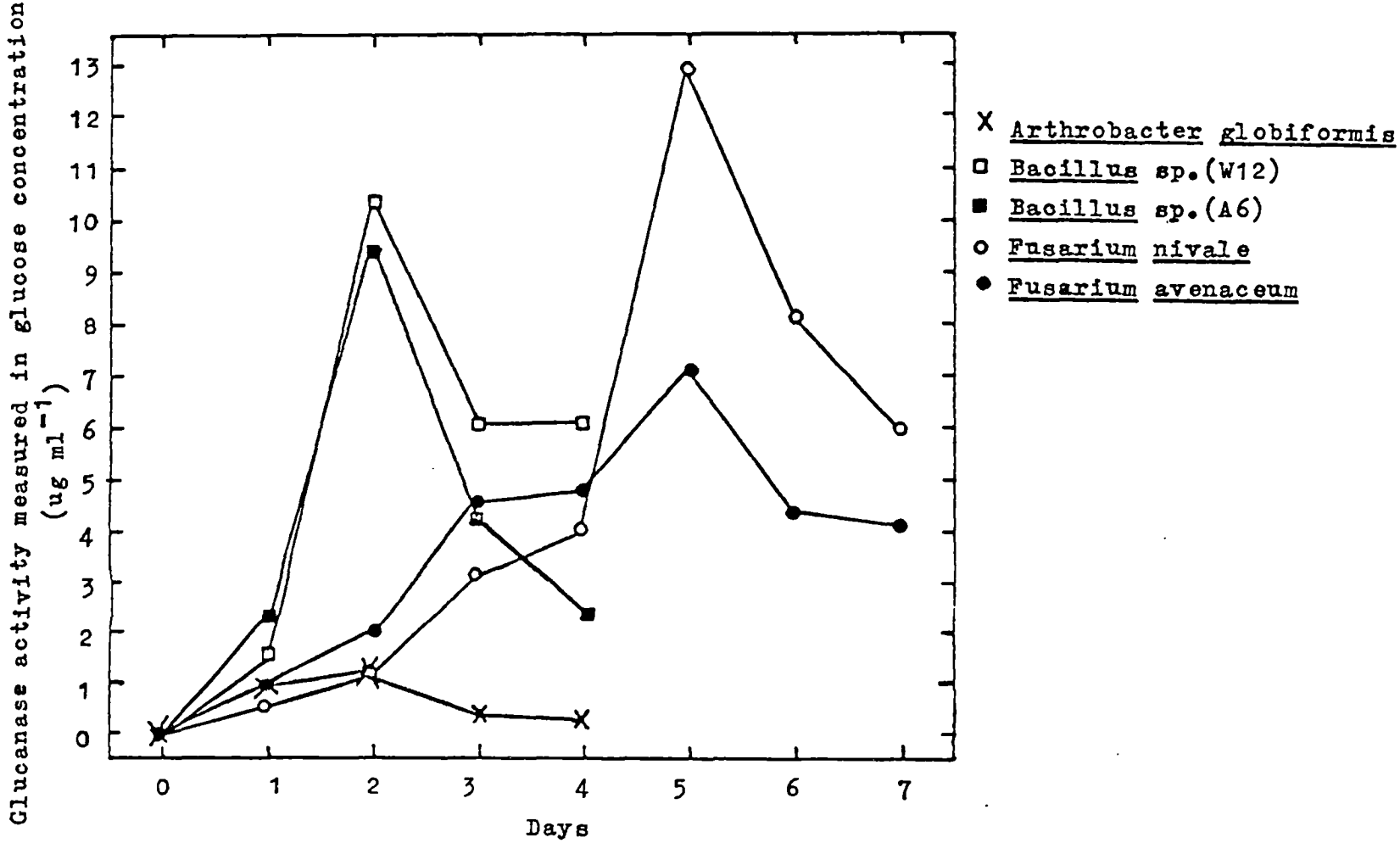


Figure 8. Effect of days of incubation on microbial production of β-glucanase at 25°C.

Table 22

Effect of days of incubation on microbial production of β-glucanase at 25° C.

Micro-organism	Degradation ($\mu g\ ml^{-1}$ glucose released 0.5 ml^{-1} extract h^{-1}) Day 1	2	3	4	5	6	7
Bacteria							
Microbacterium lacticum (A11)	0.3*	7.2	4.4	1.8	ND	ND	ND
Bacillus sp. (A6)	2.3	9.3	4.2	2.3	ND	ND	ND
Bacillus sp. (W12)	1.5	10.3	6.0	6.0	ND	ND	ND
Arthrobacter globiformis (A9)	0.9	1.2	0.3	0.2	ND	ND	ND
Microbacterium imperiale (A10)	1.1	4.4	1.9	0.5	ND	ND	ND
Fungi							
Geotrichum candidum (Fm7)	0	0	0	0	0	0	0
Fusarium avenaceum (Fm6)	1.0	2.0	4.5	4.8	7.0	4.3	4.0
Fusarium nivale (Fm5)	0.5	1.0	3.0	4.0	13.0	8.0	6.0

ND = not determined. *Figures are averages of triplicates; Inocula: bacteria = 7 x 10^5 cells ml^{-1}, fungi = 4 mm plug.

period for incubation of F. nivale and F. avenaceum. However, G. candidum did not give any detectable activity of the enzyme.

Effect of pH levels on microbial β-glucanase activity

The effect of pH levels on microbial β-glucanase activity has been included in Fig. 9 and Table 23. Two peaks were apparent at pH 5.6 and pH 7.0 for all the bacterial cultures, except Bacillus sp. (A6) which

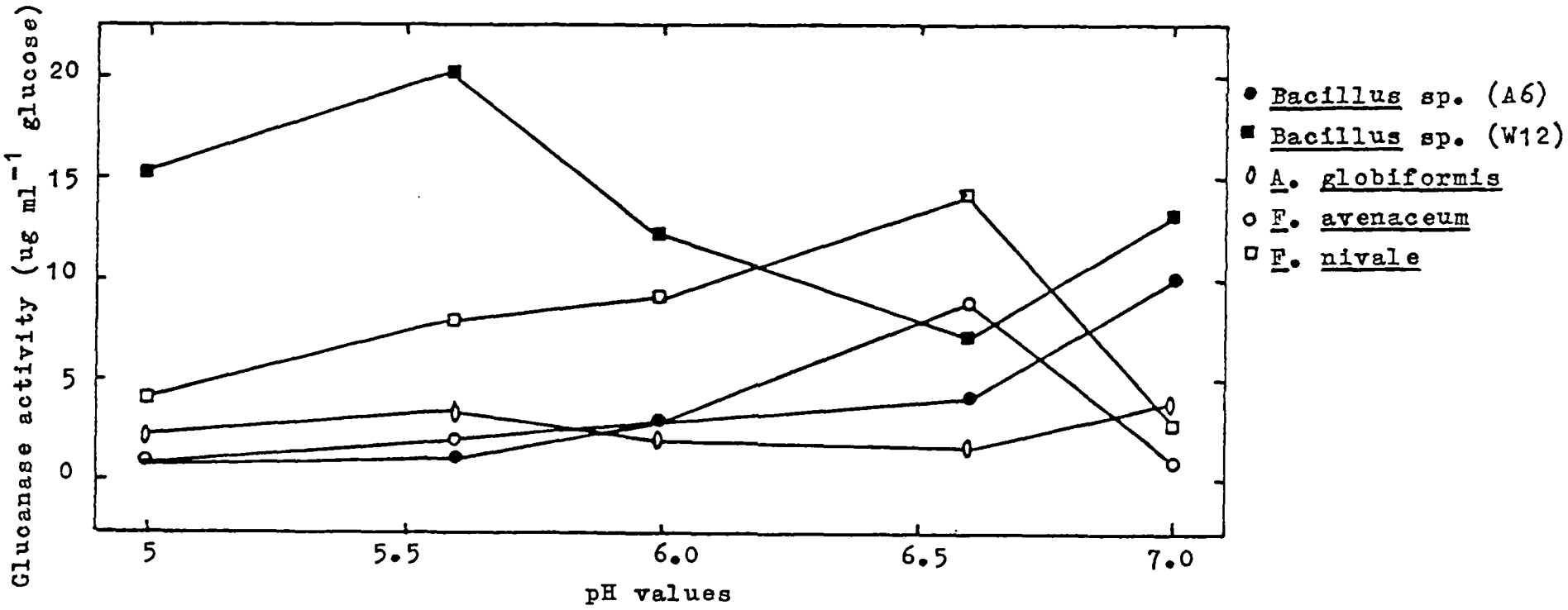


Figure 9. Effect of pH levels on degradation of β-glucan by microbial β-glucanases at 37°C.

Table 23

Effect of pH levels on degradation of barley β-glucan by β-glucanase from the micro-organisms

Micro-organism	Degradation (μg ml^{-1} glucose released 0.5 ml^{-1} extract h^{-1}) pH levels 5.0	5.6	6.0	6.6	7.0
Bacteria					
Bacillus sp. (A6)	1.0*	1.2	3.0	0.4	10.0
Bacillus sp. (W12)	15.0	20.0	12.0	7.0	13.0
Arthrobacter globiformis (A9)	1.9	3.0	2.0	1.8	3.2
Microbacterium imperiale (A10)	2.0	1.9	0.9	4.0	4.0
Microbacterium lacticum (A11)	3.0	3.0	1.5	5.2	7.6
Fungi					
Fusarium avenaceum (Fm6)	1.0	2.4	3.0	9.0	1.0
Fusarium nivale (Fm5)	4.0	8.4	9.0	14.0	3.0
Geotrichum candidum (Fm7)	0	0	0	0	0

*Figures are averages of duplicates; inocula: bacteria = 8 x 10^5 cells ml^{-1}, fungi = 4 mm plug.

had only one peak at pH 7. Apart from Bacillus sp. (W_{12}), other bacteria had higher activities at pH 7.0. The cultures of F. nivale and F. avenaceum showed their maximum activities at pH 6.6. G. candidum again did not show any detectable activity.

Reduction in viscosity of barley β-glucan caused by microbial β-glucanases at 37° C

Fig. 10 and Table 24 show reduction in viscosity of barley β-glucan brought about by microbes isolated from malting barley. Of all the bacteria and fungi tested, only F. nivale caused a noticeable change in viscosity of barley β-glucan. Approximately 19.2 units of enzyme were required to bring about a 10% reduction in 100 min (Clarke & Stone, 1962).

Effect of temperature on the growth of F. nivale and G. candidum on different solid media

On PDA and 0.1% (w/v) glucan agar, F. nivale showed a noticeable growth as from the fifth day of incubation at 4° C, whereas no growth was visible on 0.1% (W/v) starch agar even after 7 d incubation at the same temperature. This organism grew well at the temperature range of 10 - 25° C on the three media. No growth was visible on all media at 30° C and above (Table 25). G. candidum did not grow at temperatures of 4° C and above 37° C on the three media. It had fairly good growth on PDA at a temperature range of 18 - 30° C, but grew poorly at 10° and 37° C (Table 26).

Effect of temperature on production of extracellular enzymes by micro-organisms

On plates, cultures of F. nivale showed amylolytic activity between the temperatures of 10 and 25° C, but glucanase activity between 18 and 25° C. G. candidum did not produce any detectable activity even though it grew at temperatures between 10 and 30° C (Table 27).

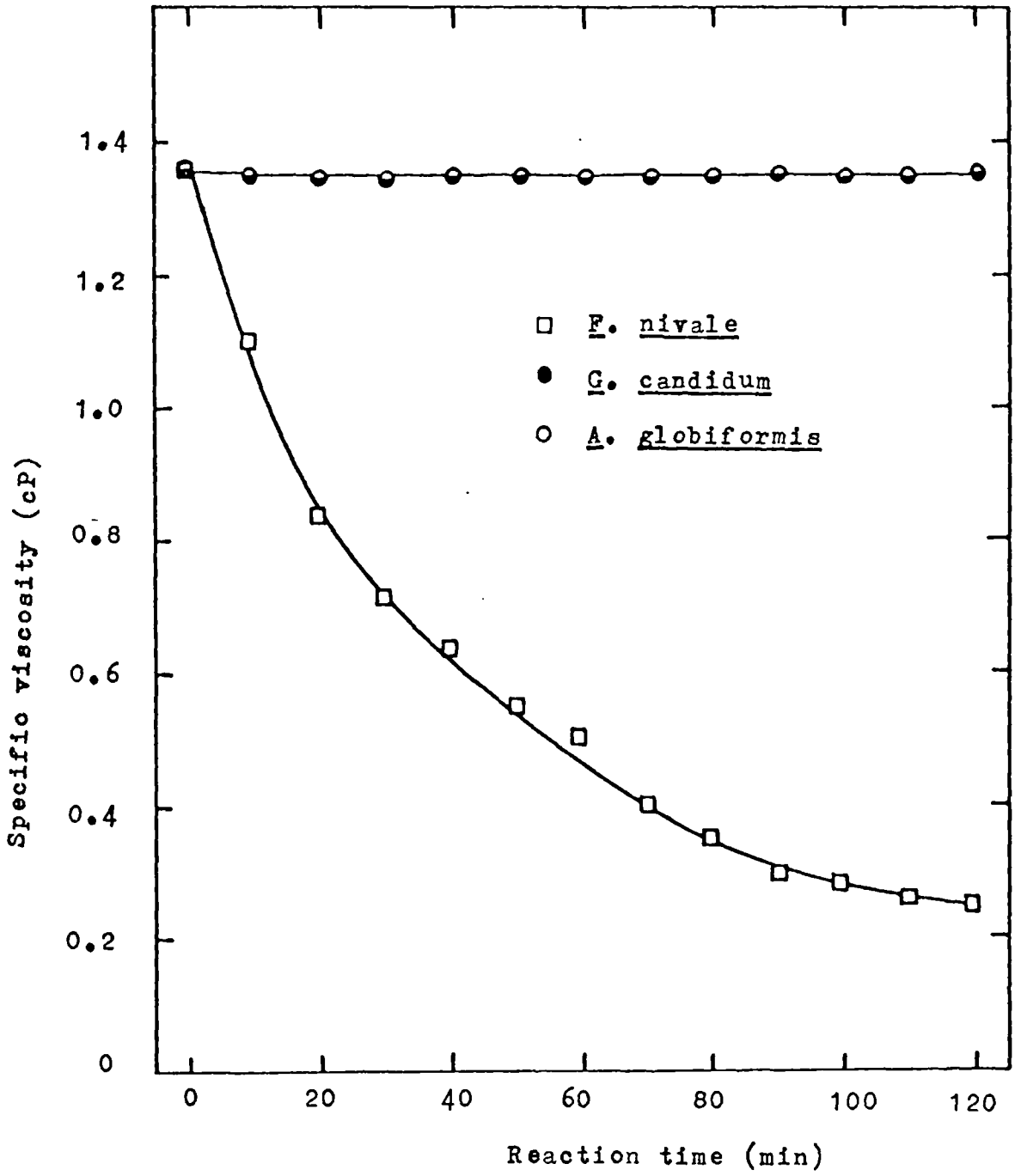


Figure 10. Estimation of microbial β-glucanase reducing power at 37°C, using a Brookfield viscometer. (Assay: 3ml of 0.5% glucan + 0.15ml culture filtrate).

Table 24

Reduction in viscosity of barley β-glucan caused by microbial β-glucanases at 37° C, using a Brookfield viscometer.

Organism	Time (min.)	Sp. viscosity
F. nivale (Fm5)	0	1.36
	10	1.10
	20	0.84
	30	0.71
	40	0.64
	50	0.55
	60	0.45
	70	0.40
	80	0.35
	90	0.30
	100	0.28
	110	0.21
	120	0.15

Note: The following organisms showed no detectable levels of reduction in viscosity: Microbacterium lacticum (A11), M. imperiale (A10), Bacillus sp. (A6 and W12), Arthrobacter globiformis (A9), Fusarium avenaceum (Fm6), and Geotrichum candidum (Fm7). Assay: 3 ml substrate (0.5% w/v) + 0.15 ml enzyme filtrate.

Table 25

Growth of Fusarium nivale (Fm5) at various temperatures and at different incubation periods on plates.

Temp. of incubation °C	Medium	Diameter of Growth (mm) Days 1	2	3	4	5	6	7
4	PDA	0	0	0	0	6.0	7.0	10.0
10	PDA	0	0	7.0	12.0	16.3	19.7	22.3
18	PDA	0	17.7	29.7	39.3	51.3	67.0	72.7
20	PDA	7	14.3	39.0	54.0	69.3	84.7	>90.0
25	PDA	9.7	19.7	44.0	58.7	75.7	90.0	>90.0
30	PDA	0	0	0	0	0	0	0
37	PDA	0	0	0	0	0	0	0
50	PDA	0	0	0	0	0	0	0
60	PDA	0	0	0	0	0	0	0
4	0.1% glucan agar	0	0	0	0	6	7	12
10	0.1% glucan agar	0	0	7.0	19.3	23.7	28.3	33.0
18	0.1% glucan agar	0	12.3	30.0	39.3	48.7	55.3	59.7
20	0.1% glucan agar	0	12.7	31.3	40.7	48.0	57.7	64.0
25	0.1% glucan agar	8	14.3	32.0	40.7	52.3	55.7	55.7
30	0.1% glucan agar	0	0	0	0	0	0	0
37	0.1% glucan agar	0	0	0	0	0	0	0
50	0.1% glucan agar	0	0	0	0	0	0	0
60	0.1% glucan agar	0	0	0	0	0	0	0
4	0.1% starch agar	0	0	0	0	0	0	0
10	0.1% starch agar	0	10.0	14.0	21.3	29.0	35.3	44.7
18	0.1% starch agar	8	15.0	22.3	31.7	45.0	56.7	62.3

Table 25 (cont.)

Temp. of incubation °C	Medium	Diameter of Growth (mm) Days 1	2	3	4	5	6	7
20	0.1% starch agar	9.0	22.3	35.7	48.7	62.0	70.0	73.3
25	0.1% starch agar	9.0	21.3	34.0	43.7	57.3	66.3	67.7
30	0.1% starch agar	0	0	0	0	0	0	0
37	0.1% starch agar	0	0	0	0	0	0	0
50	0.1% starch agar	0	0	0	0	0	0	0
60	0.1% starch agar	0	0	0	0	0	0	0

Note: Inoculum = 4 mm agar plug of 5 d culture.

Table 26

Growth of Geotrichum candidum (Fm7) at various temperatures and at different incubation periods on plates.

Temp. of incubation °C	Medium	Diameter of Growth (mm) Days 1	2	3	4	5	6	7
4	PDA	0	0	0	0	0	0	0
10	PDA	0	0	9.0	11.7	13.3	15.0	18.0
18	PDA	9	11.3	15.0	18.3	23.7	25.7	30.7
20	PDA	11.0	16.0	21.3	25.3	32.7	38.7	43.7
25	PDA	11.0	19.0	27.3	34.3	42.7	52.7	54.0
30	PDA	14.0	25.0	37.0	47.3	57.3	70.3	79.7
37	PDA	11	10.7	12.0	12.0	12.0	12.0	12.0
50	PDA	0	0	0	0	0	0	0
60	PDA	0	0	0	0	0	0	0
4	0.1% starch agar	0	0	0	0	0	0	0
10	0.1% starch agar	0	0	9.0	10.0	11.0	12.0	13.0
18	0.1% starch agar	9.0	10.3	13.0	15.0	18.7	19.7	22.3
20	0.1% starch agar	9.0	12.7	15.0	18.7	22.0	25.7	29.0
25	0.1% starch agar	10.3	16.0	20.0	23.0	27.0	32.0	35.0
30	0.1% starch agar	11.3	19.3	24.3	29.3	33.7	37.7	41.7
37	0.1% starch agar	0	0	0	0	0	0	0
50	0.1% starch agar	0	0	0	0	0	0	0
60	0.1% starch agar	0	0	0	0	0	0	0
4	0.1% glucan agar	0	0	0	0	0	0	0
10	0.1% glucan agar	0	0	0	9	9	10.0	10.0
18	0.1% glucan agar	9.0	10.1	10.3	12.0	12.5	13.1	13.1

Table 26 (cont.)

Temp. of incubation °C	Medium	Diameter of Growth (mm) Days 1	2	3	4	5	6	7
20	0.1% glucan agar	9.0	11.0	11.0	12.5	12.8	13.2	13.8
25	0.1% glucan agar	9.0	11.0	12.0	12.9	14.7	14.9	15.1
30	0.1% glucan agar	9.0	11.5	11.8	13.9	15.6	16.5	16.7
37	0.1% glucan agar	0	0	0	0	0	0	0
50	0.1% glucan agar	0	0	0	0	0	0	0
60	0.1% glucan agar	0	0	0	0	0	0	0

Note: Inoculum = 4 mm agar plug of 5 day culture.

Table 27

Growth and enzymatic activities of *F. nivale* and *G. candidum* at various temperatures after 2 - 7 d incubation on 0.1% starch plates.

	F. nivale				*G. candidum*	
Temp. of incubation °C	Growth diameter (mm)	Glucanase activity	Growth diameter (mm)	Amylolytic activity	Growth diameter (mm)	Amylolytic activity
4	12	-	0	ND	0	ND
10	33	-	44	+	13	ND
18	59	+	62	+	22	-
20	64	+	73	+	29	-
25	56	+	67	+	35	-
30	0	ND	0	ND	41	-
37	0	ND	0	ND	ND	ND
50	0	ND	0	ND	ND	ND
60	0	ND	0	ND	ND	ND

ND = not determined; - = negative response; + = positive response.

Effect of temperature on extracellular enzyme activity

Culture filtrate from F. nivale showed glucanase activity on β-glucan at a temperature range of 10 - 50° C, with an increase as the temperature rose (Fig. 11).

The same trend was observed in the case of amylase activity for culture filtrate from F. nivale (Fig. 11 and Table 28). Amylase activity rose with increasing temperature.

Effect of incubation period on glucanase activity at 37° C in liquid medium, using enzyme filtrate from F. nivale

Fig. 12 and Appendix 13 show that the activity of glucanase rose linearly with time at for the first 50 - 100 min, after which the rise was no longer linear. After a stationary phase at approximately 12 h, the activity started to decrease as time increased. However, the curve was not smooth after about 2 h incubation.

Alpha-amylase activity

Production of α-amylases by F. nivale and G. candidum results are shown in Fig. 13a, b and Tables 29 and 30. There was a rapid reduction in iodine staining power of β-limit dextrin during the first 20 min. Thereafter the reduction was gradual as time increased (Fig. 13a). Culture filtrate from F. nivale brought about a reduction in iodine staining power of β-limit dextrin with quantities as low as 0.1 ml of the filtrate (Fig. 13b and Table 30). G. candidum cultures, in contrast did not cause any reduction in iodine staining power of β-limit dextrin. Since α-amylase is regarded as specific for hydrolysis of β-limit dextrin, it therefore means that F. nivale produced α-amylase which G. candidum did not produce under the conditions of study.

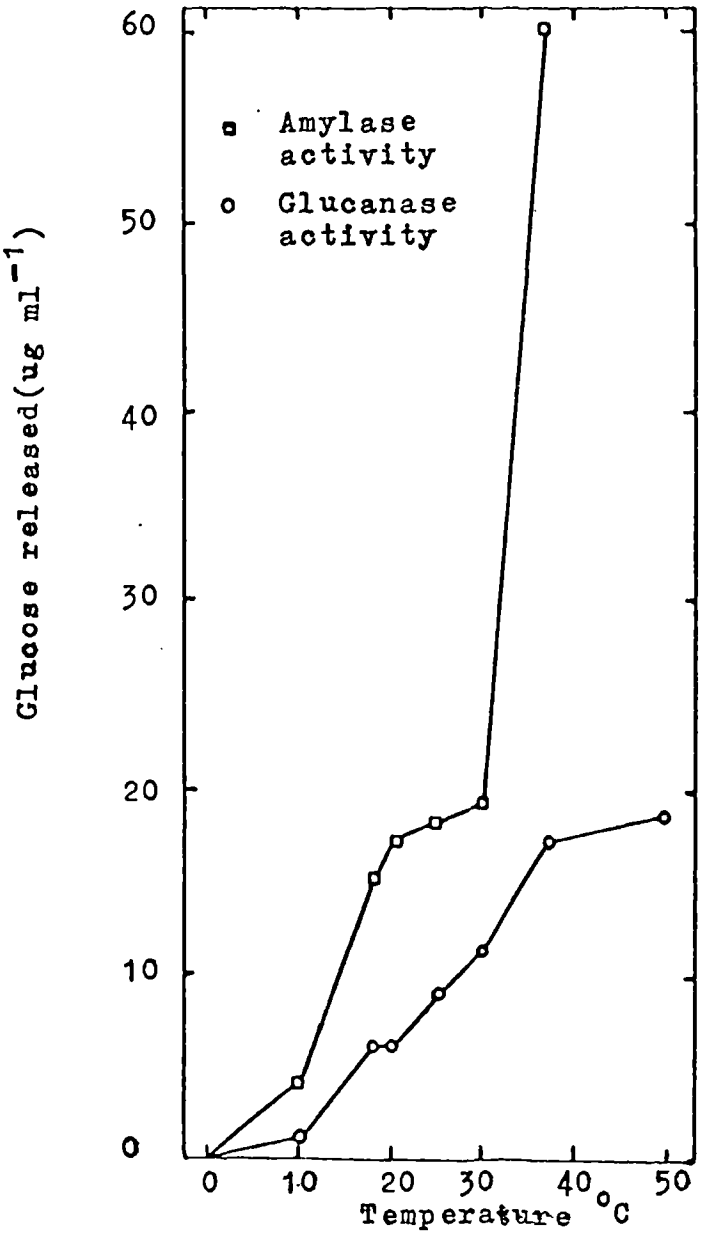


Figure 11. Effect of temperature on Fusarium nivale extracellular enzyme activity.

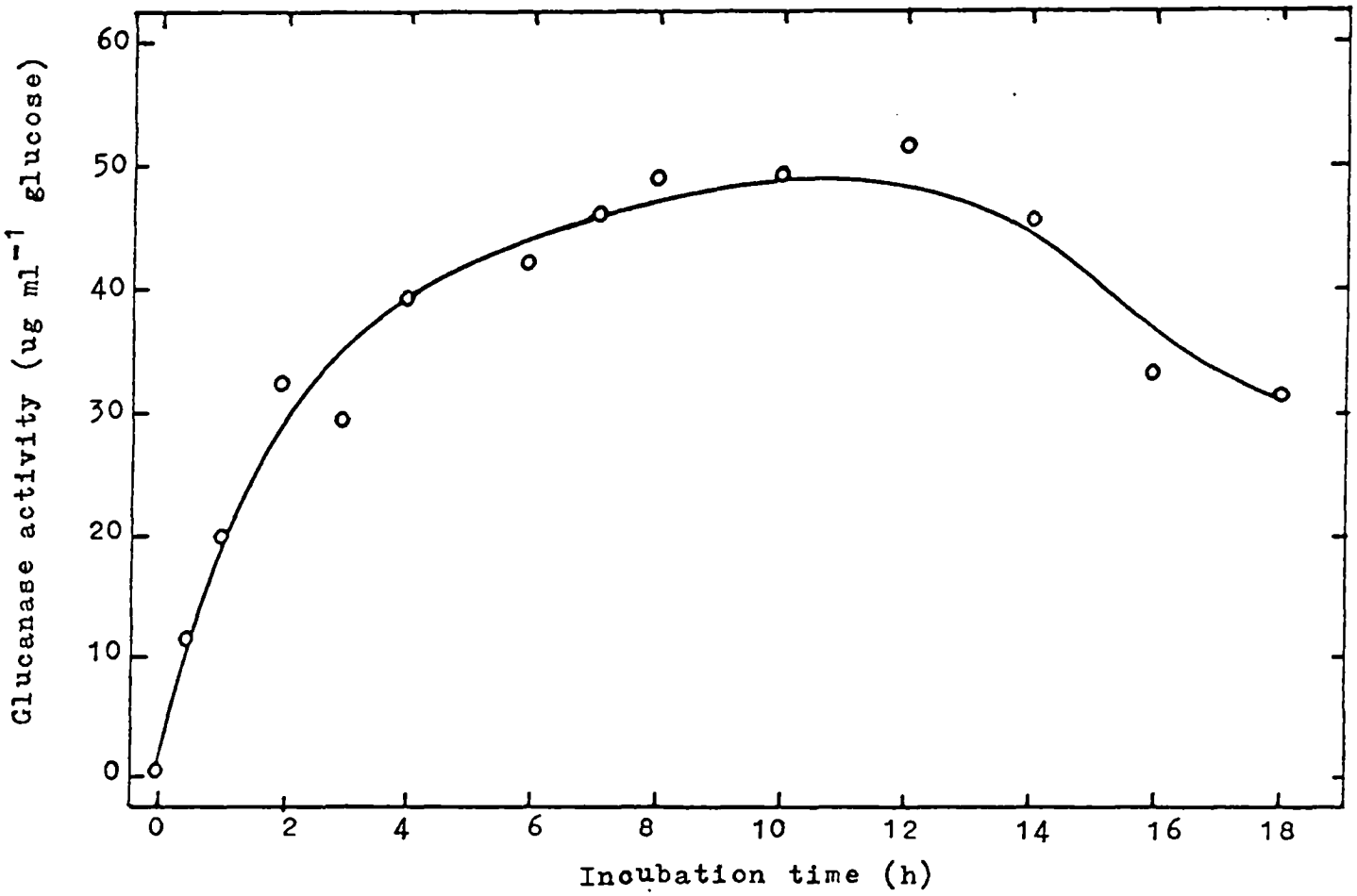


Figure 12. Effect of length of incubation of culture filtrate of F. nivale on glucanase activity at 37°C

Table 28

Effect of temperature on enzymatic activity using enzyme filtrate from *F. nivale* at pH 6.6.

Temperature (°C)	Enzyme Activity ($\mu g\ ml^{-1}$ glucose released 0.5 ml^{-1} extract 2 h^{-1})	
	β-glucanase	Amylase
0	0	0
10	1.2	4.0
18	6.0	15.0
20	6.2	17.0
25	9.0	18.0
30	11.0	19.0
37	17.0	60.0
50	18.5	ND

ND = not determined.

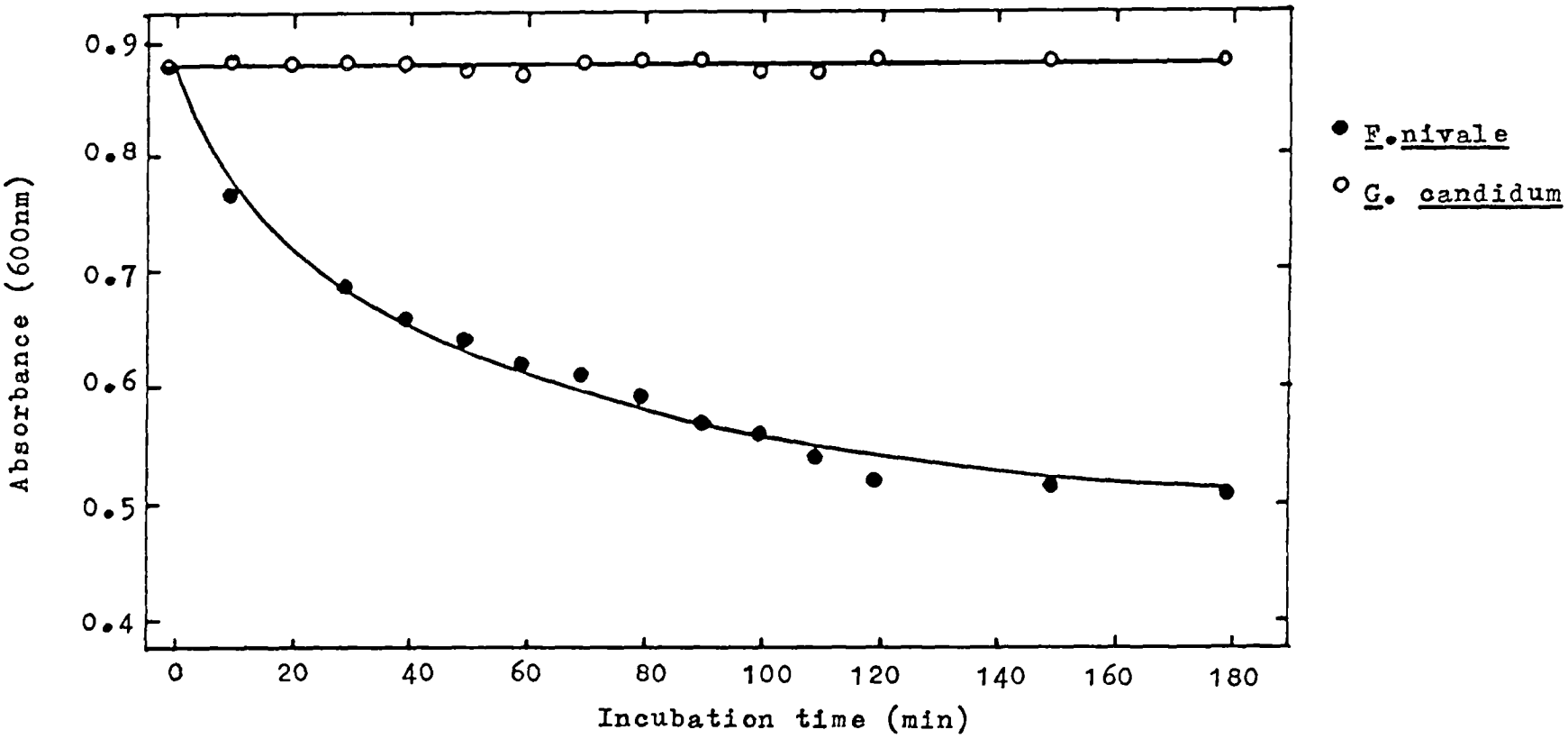


Figure 13a. Effect of length of incubation of culture filtrate of F. nivale and G. candidum on iodine staining in β-limit dextrin.

Table 29

Effect of length of incubation of culture filtrates of F. nivale and G. candidum on iodine staining in β-limit dextrin.

Incubation Time (min)	Absorbance (600 nm)	
	F. nivale	G. candidum
0	0.88	0.88
10	0.77	0.88
20	0.72	0.88
30	0.69	0.88
40	0.66	0.88
50	0.64	0.87
60	0.62	0.87
70	0.61	0.88
80	0.59	0.88
90	0.57	0.87
100	0.56	0.87
110	0.54	0.87
120	0.52	0.88
150	0.52	0.88
180	0.51	0.88

Assay: 0.5 ml substrate + 0.5 ml culture filtrate at 37° C.

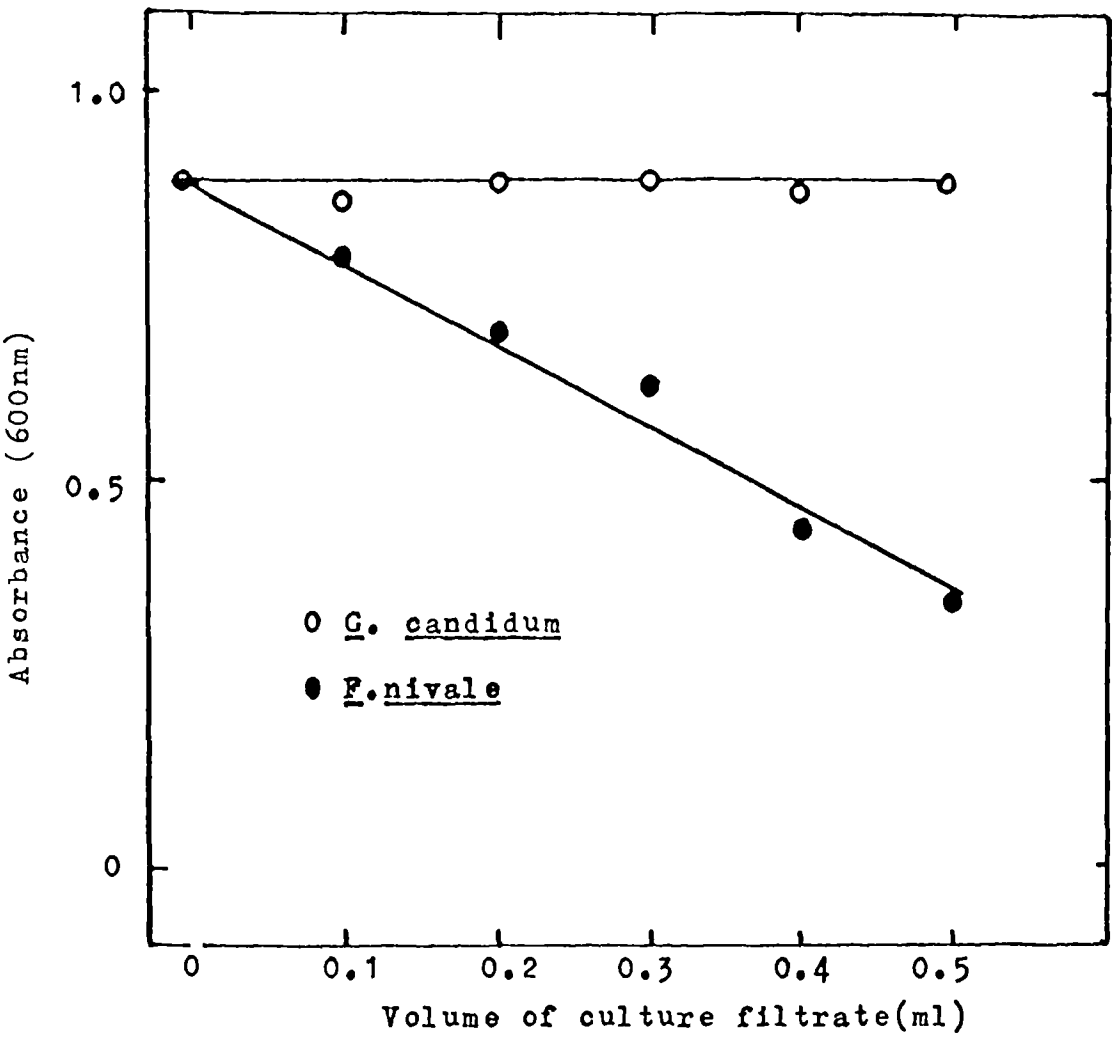


Figure 13b. Effect of concentration of culture filtrate of F. nivale and G. candidum on iodine staining in β-limit dextrin.(Assay: at 37°C for 10min).

Table 30

Effect of concentration of culture filtrates of F. nivale and G. candidum on iodine staining in β-limit dextrin.

Enzyme source (dialysed)	Absorbance (600 nm) Volume of culture filtrate (ml) 0.0 (blank)	0.1	0.2	0.3	0.4	0.5
F. nivale	0.88	0.79	0.69	0.62	0.43	0.34
G. candidum	0.88	0.86	0.87	0.88	0.87	0.88

Assayed at 37° C for 10 min.

The relationship between amylolytic activities of F. nivale, measured by reduction in iodine staining power of soluble starch and that by reducing power (μg ml^{-1} glucose) is depicted in Fig. 14 and Table 31.

Raffinose and sucrose degradation

Since barley contains other carbohydrates besides β-glucan and starch, culture filtrates from F. nivale and G. candidum were reacted with raffinose and sucrose to examine for potential degradation by fructosidase. The results obtained showed that F. nivale degraded raffinose and sucrose, but G. candidum did not (Fig. 15 and Table 32).

Protein content in culture filtrate of 5 d mould cultures at 25° C

Despite the non-reactivity of G. candidum culture filtrates in the present study, substantial amounts of protein (0.71 mg ml^{-1}) comparable to those of F. nivale (0.7 - 0.8 mg ml^{-1}) (Table 33) were present.

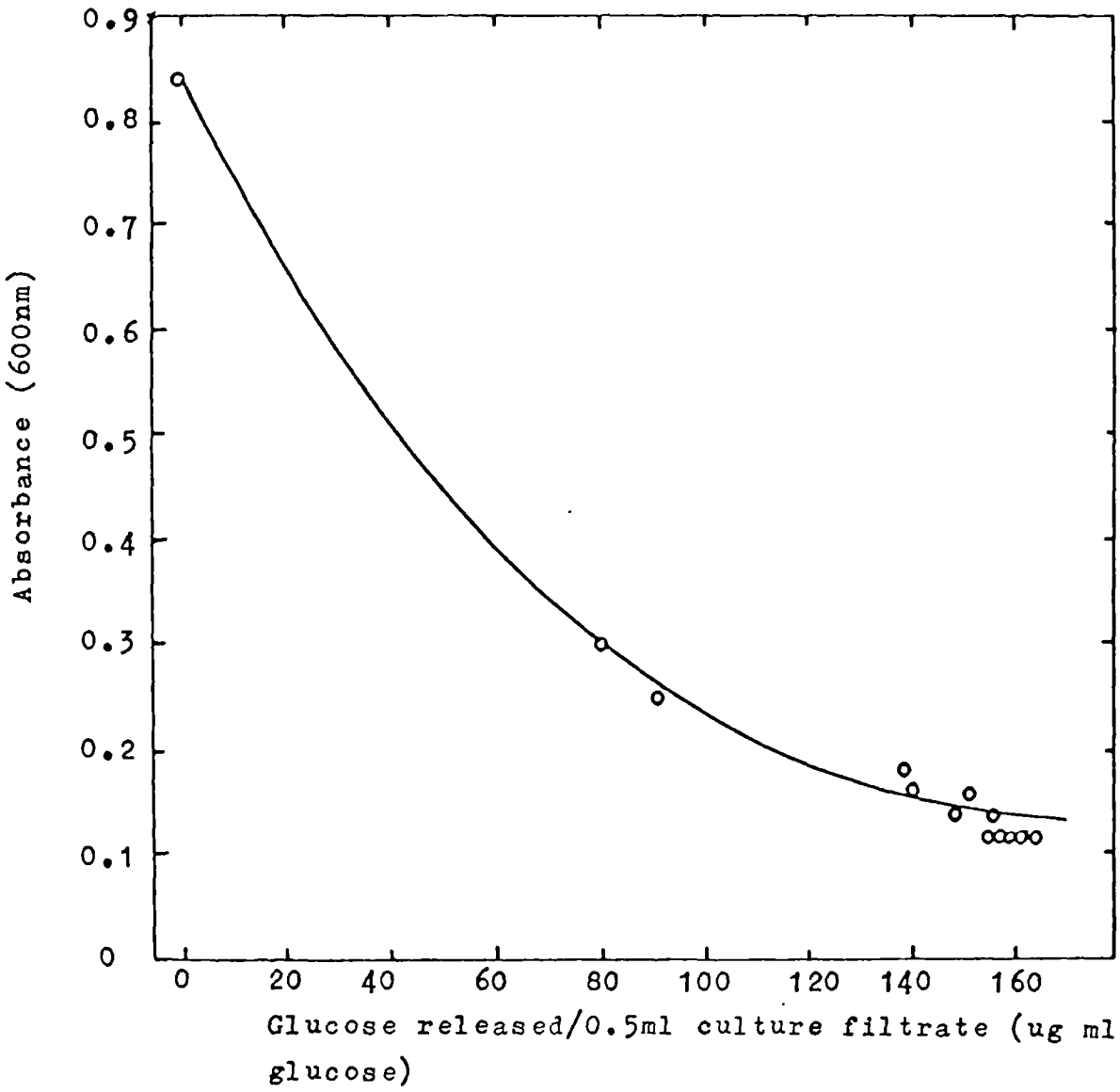


Figure 14. Relationship between amylolytic activity of F. nivale measured by reduction in iodine staining(absorbance) and by reducing sugar levels.

Table 31

Amylolytic - activity of F. nivale and G. candidum at 37° C, using reduction in iodine staining and reducing sugar levels.

	F. nivale		G. candidum	
Incubation time (min)	I_2-staining absorbance (600 nm)	Reducing sugars ug ml^{-1} glucose released $0.5ml^{-1}$ extract	I_2-staining absorbance (600 nm)	Reducing sugars ug ml^{-1} glucose released $0.5ml^{-1}$ extract
0	0.84	0.0	0.85	0.0
10	0.30	81.0	0.85	0.0
20	0.25	91.0	0.82	0.0
30	0.21	138.0	0.83	0.0
40	0.17	140.0	0.83	0.0
50	0.16	151.0	0.85	0.0
60	0.14	149.0	0.85	0.0
70	0.14	156.0	0.85	0.0
80	0.12	153.0	0.85	0.0
90	0.12	156.0	0.84	0.0
100	0.12	158.0	0.84	0.0
110	0.12	159.0	0.85	0.0
120	0.12	161.0	0.85	0.0
150	0.12	164.0	0.85	0.0
180	0.12	164.0	0.85	0.0

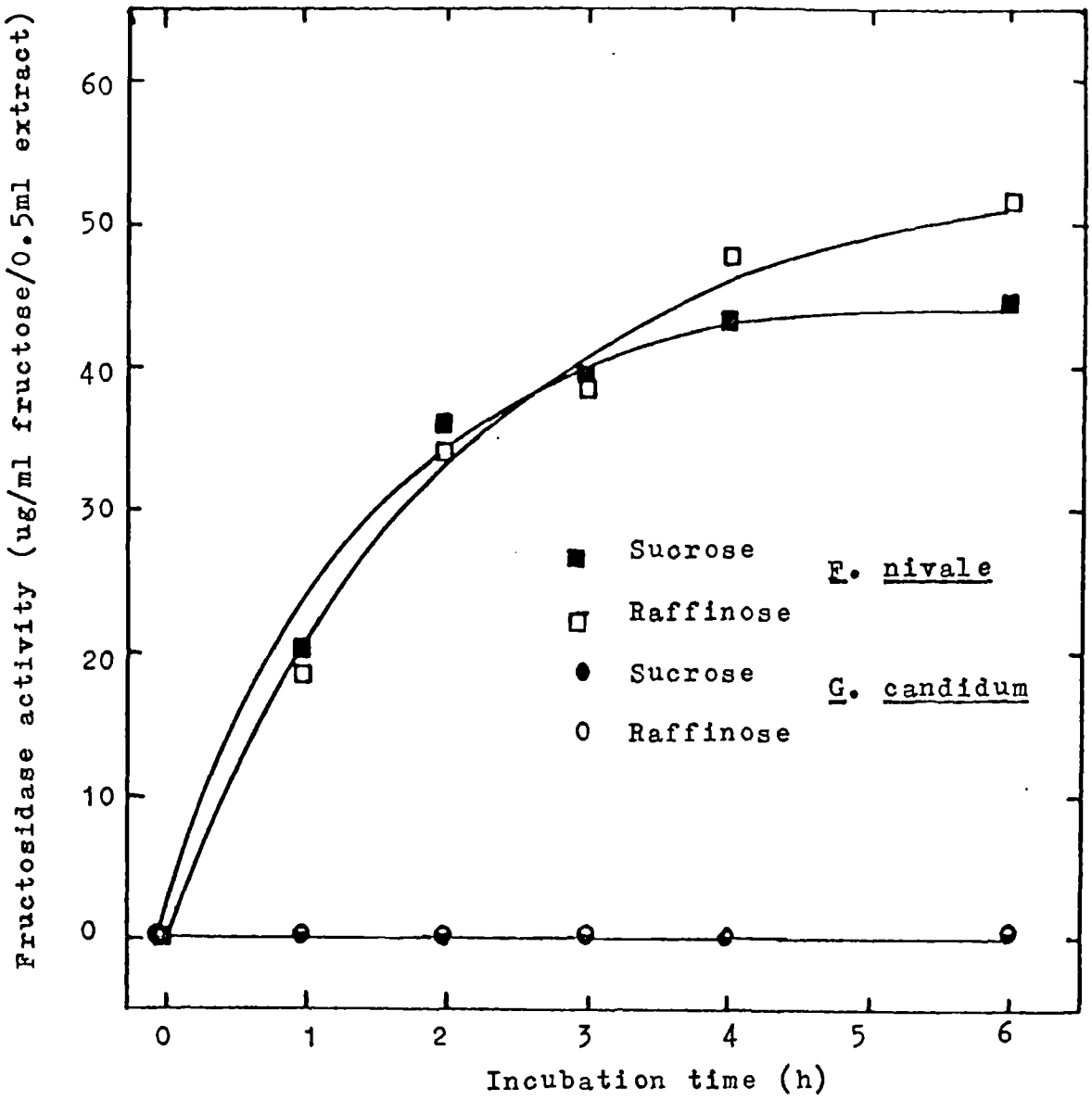


Figure 15. Degradation of raffinose and sucrose by F. nivale and G. candidum at 37°C. (Assay: 0.5ml culture filtrate + 0.5ml substrate in acetate buffer of pH 5.6).

Table 32

Degradation of raffinose and sucrose by F. nivale and G. candidum at 37° C.

Incubation time (h)	F. nivale		G. candidum	
	OD (600 nm)	Activity µg ml^{-1} glucose	OD (600 nm)	Activity µg ml^{-1} glucose
Substrate: sucrose				
0	0	0	0	0
1	0.15	20.0	0	0
2	0.27	36.0	0	0
3	0.29	39.0	0	0
4	0.32	43.0	0	0
6	0.33	44.0	0	0
Substrate: raffinose				
0	0	0	0	0
1	0.13	19.0	0	0
2	0.25	34.0	0	0
3	0.28	38.0	0	0
4	0.35	47.0	0	0
6	0.39	51.0	0	0

Assay: 0.5 ml culture filtrate + 0.5 ml substrate, pH 5.6.

Table 33

Protein content in culture filtrate of 5 d mould cultures at 25° C

Source	Protein (mg ml^{-1})
F. nivale, grown in glucose medium	0.70
F. nivale, grown in sucrose medium	0.80
F. nivale, grown in raffinose medium	0.71
G. candidum, grown in glucose medium	0.71
G. candidum, grown in sucrose medium	0.71
G. candidum, grown in raffinose medium	0.71

DISCUSSION

Micro-organisms of Malting Barley

As mentioned in the introduction, despite the economic importance of barley and malt products to the brewing, distilling and food industries, relatively little has been published about the microbiology of modern malting procedures. In recent years, technological advances have reduced the time required to produce well-modified malt, and this reduction, together with the burning of sulphur to combat the formation of nitrosamines during kilning (Flannigan, 1983), has important implications for microbial development and survival. Indeed, knowledge of the nature, magnitude and distribution of microbial populations in malting barley ecosystems must be considered as incomplete without a detailed investigation of commerical malting processes. Unfortunately, most of the microbiological investigations to date have involved merely material derived from laboratory or micro-malting systems (Flannigan, 1987). Whereas laboratory malting may give a broad indication of microbiological changes which occur during the process, extrapolation from laboratory to full-scale commercial malting is unwise. It is relevant to note that marked differences in the development of microbial numbers during germination have been reported between laboratory and commercial malting (Flannigan *et al.*, 1982). Moreover, most studies have been concerned mainly with fungi, whereas detailed studies on the bacterial flora have remained in abeyance. The present detailed study of the microflora of malting barley, during a modern commercial operation will be useful in understanding, more clearly, the precise role of such organisms in the process. This aspect will be considered later.

Although it would have been desirable to study several commercial production runs in order to draw firm conclusions, it was decided to examined one batch in detail. The information so obtained will be used to compare the microbiology of further commercial runs in the future. An alternative strategy to use fewer strains from a multiplicity of production runs would have been of questionable statistical relevance (Wardlaw, 1985). Yet to take a meaningful number of microbial strains from more samples would have generated an intolerable level of work. Nevertheless, the overall significance of the data obtained in this study is reinforced by its relevance to previous publications.

In the present study, the numbers of micro-organisms were calculated for single kernels rather than for unit weights, i.e. per gram as proposed by earlier researchers. The use of this approach stemmed from the fact that it offers a sensible comparative basis (Flannigan, 1977) between samples from different situations, (in this case, at different stages of malting) since weights of barley corns vary. Despite the difference from gravimetric approach, comparison can still be made with earlier studies. Thus, the numerical dominance of aerobic heterotrophic, mesophilic bacteria, and the presence of proportionally (and sequentially) smaller populations of yeasts and filamentous fungi, is in overall agreement with previous work on stored barley (Hill & Lacey, 1983a). In malting barley, Douglas & Flannigan (1988) recorded the highest population of 1.2×10^8 (mesophilic bacteria), 2.0×10^6 (yeasts), and 1.4×10^4 (moulds) $kernel^{-1}$ during germination, whereas in this study, the corresponding numbers were 5.7×10^7, 3.9×10^6 and 1.7×10^3. However, the highest population in the case of moulds was on the steeped out grain. The reason for the low count of filamentous fungi at the green malt stage is not readily apparent, but

the possibility of antagonistic interactions between micro-organisms cannot be overlooked. For example, the numbers of Fusarium nivale have been observed to decline in the presence of Epicoccum (Limonard, 1968).

Whereas some workers have reported substantial increases in bacterial counts of up to 37- and 18-fold on bright and weathered barley (Kotheimer & Christensen, 1961) and 7-fold (Haikara et al., 1977) during steeping in laboratory maltings, only a slight increase was reported in commercial malting (Sheneman & Hollenbeck, 1960; Kneen, 1963). Conversely in the present study, a slight decrease was observed on the first steeped grain, with a subsequent slight increase on steeped out grain. This decrease may be explained by the fact that steep-water might have washed substantial numbers of bacteria off the grain. This was substantiated by the observation that such waters contained high numbers of bacterial cells. The progressive increase in microbial populations from dried barley to green malt is undoubtedly a function of the enhanced nutrient levels during hydration and germination. The decline in microbial populations with kilning confirms earlier observations (Sheneman & Hollenbeck, 1960; Douglas & Flannigan, 1988). Data for mould populations conflict with previous work. The lowest mould count on green malt contrasts with observations of other workers. The subsequent increase in mould populations with kilning and on screened malt may be explained by the activation of propagules by the kilning process. Indeed, some moulds, namely Mucor and Rhizopus, have been reported to multiply during kilning (Haikara et al., 1977; Gyllang & Martinson, 1976b).

Overall, the population trends observed in this study follow those in previous work. For example, the bacterial populations on green and kilned malt, i.e. 5.7×10^7 kernel^{-1} and 5.6×10^6 kernel^{-1}

respectively, fall well within the range stated by Flannigan et al. (1982) and, by calculation from gravimetric results, Boruff et al. (1938) and Sheneman & Hollenbeck (1960). Generally, any differences in the microbial population reflect differences in the precise nature and preparation of the sample, i.e. whether the malt is destined for distilling or brewing. Where it is practised, sulphuring during kilning has been shown to cause marked reductions in microbial numbers, either when used to bleach malt (Graff, 1972) or to prevent nitrosamine formation (Flannigan, 1983). Moreover, it is apparent that the nature of the malting process influences the composition of the microflora. For example, the proportional increases in the numbers of bacteria and filamentous fungi are greater in floor maltings than procedures using saladin boxes. Conversely, yeast development is favoured by the latter (Flannigan et al., 1982).

The results from this study and from previous work, e.g. Sheneman & Hollenbeck, 1960, indicated that lactic acid bacteria, notably Lactobacillus spp., are a constant component of the natural microflora of barley during malting. In this investigation, lactobacilli occurred on all grain types from dried barley to screened malt. Such organisms represented approximately 15% of the total aerobic, heterotrophic bacterial population on the 5-day green malt. This proportion is higher than the report of Sheneman & Hollenbeck (1960), who recorded <10% lactobacilli. Even screened malt harboured substantial numbers of micro-organisms, an observation which is in accord with previous work (Boruff et al., 1938; Flannigan et al., 1982).

Although Sheneman & Hollenbeck (1960) noted that populations of thermophilic lactic acid bacteria, i.e. those recovered by incubation at 45° C, remained very low throughout malting, the experience from

this study is that such organisms are not recovered at incubation temperatures of >50° C. Conversely, there appeared to be no psychrophilic lactic acid bacteria, i.e. with incubation at <15° C.

There was no evidence of psychrophilic or thermophilic filamentous fungi. This is not so surprising, however, as most moulds grow normally within mesophilic temperatures. Yet thermophilic fungi, such as Mucor (Rhizomucor) pusillus and Thermomyces lanuginosus, have been reported to develop in hot spots of 65° C (Lacey & Hill, 1983b). However, the recovery of micro-organisms on kilned malt does not necessarily indicate that these organisms are thermophiles, which survived the kilning temperature. It seems likely that recontamination is possible after kilning, as may be the explanation for recovery of mesophiles. The absence of thermophilic yeasts is explained by the fact that they do not survive the mashing temperature (Campbell, 1987).

Certainly, the presence of bacteria and filamentous fungi between the enveloping husk and the underlying seed coat of pre-harvest barley has been well documented, albeit without the supporting evidence of electron microscopy, e.g. Warnock (1973). The observation of fungal hyphae on the inner, and other surfaces of kilned malt and dried barley largely confirms the work of Warnock (1973). In addition, numerous bacterial cells and (what were most probably) yeasts were observed on the outer surfaces of the barley/husk, with less cells in the inner surface. This may be explained by the fact that the outer surfaces of the barley are exposed to aerial contamination. This agrees with the observation (Hill & Lacey, 1983a) that barley kernels are contaminated with components of the airborne flora in the field. They further noted that newly emerged ears of barley are colonised by micro-organisms with bacteria being numerically dominant. The presence of micro-organisms

below the surface of the lemma and palea supports the suggestion that the pericarp layer of the caryopsis itself may also be extensively invaded by mycelium (Mead, 1943; Warnock & Preece, 1971) and perhaps less so than the lemma and palea (Tuite & Christensen, 1955).

Numerical taxonomy methods have been successfully employed in the investigation of the relationships among members of specific bacterial taxa, and in the determination of bacterial populations which are associated with a given environment or ecological function. It is the latter aspect that is of interest here. The application of numerical phenetic techniques has grown steadily since the original publication of Sneath (1957). Indeed, a wide range of habitats have been examined using numerical taxonomy including fresh water (Holder-Franklin et al., 1981), estuaries and sea water (Austin et al., 1979a; Austin et al., 1979b), leaf surfaces (Austin et al., 1978; Ercolani, 1978), and soil (Goodfellow, 1969; Graham, 1964; Rovira & Brisbane, 1967; Lowe & Gray, 1972). Results of large-scale numerical taxonomy studies have shown that, if strains possessing independent and correlated characters are recovered, then numerical methods can detect and define relationships among the constituent taxa (e.g. Austin et al., 1978; Austin, 1982). The present findings reinforce the view that numerical taxonomy methods will effectively record variation in large mixed populations of bacteria. In this first numerical phenetic study of bacteria involved with the malting of barley, 86% of the strains were recovered in 28 distinct phenetic groups. Moreover, there was sufficient consistency within, and between, phena for characters to be weighted for identification. It is unfortunate that only four (10%) of the marker strains were recovered in defined phena. This result, like those of previous studies, e.g. Austin et al. (1978), highlight the difficulties

in selecting strains for the identification of groups in analyses of large mixed populations of bacteria. As more is learned about the species composition of bacteria involved in the malting process, however, the easier the choice of marker strains will become. Undoubtedly future studies on bacteria from barley malt production should include bona fide representatives of Alcaligenes, Arthrobacter, Aureobacterium, Bacillus, Brevibacterium, Chromobacterium, Clavibacter, Corynebacterium, Erwinia, Flavobacterium, Lactobacillus, Microbacterium, Oerskovia, Pseudomonas and Serratia. It is a typical response to large scale taxonomic studies that phena, which do not contain marker strains, are often identified using conventional schemes, e.g. Austin et al. (1978). This practice may be of limited value, as to some extent noted in the present study, because distinct phena may be identified to the same taxon. These difficulties emphasize that environmental samples may contain bacteria which have been previously unrecognised or classified into heterogenous groups.

Test error in the present study was calculated to be 3.7%, i.e. within the range usually described for large-scale numerical studies, and comparable with the results of others. For example, Broom & Sneath (1981) determined the test error to be 5% in their study of bacteria belonging to the genus Haemophilus. In the taxonomic study of heterotrophic halophilic and non-halophilic bacteria from a solar saltern, the test error was calculated as 3.5% (Marquez et al., 1987). Austin (1982), in an examination of bacteria recovered from a coastal marine fish-rearing unit, reported a test error of 3.6%. In another study of Gram-positive organisms, Goodfellow et al. (1982) and Orchard & Goodfellow (1980) reported test errors of 2.5% and 7.1%, respectively. The importance of determining the test error in

numerical studies should not be underestimated, because a large error may distort similarity coefficients, and subsequently lead to misinterpretation of the data (Sneath & Johnson, 1972). However, according to Sneath & Johnson (1972), if the probability of test error does not exceed 10%, it is within acceptable limits and the validity of the data need not be questioned. Also if well-defined and homogenous phena have been recovered, this would indicate that the test error was within acceptable limits.

Little is known about the species composition of bacterial populations occurring in the malting of barley. The dominance of chromogenic bacteria, as observed in this study is in good accord with previous work on plant material, e.g. Dickinson et al. (1975), Austin et al. (1978). The range of taxa recovered in this study matches closely with previous observations on a wide range of plants, including barley malt. Thus, the presence of Alcaligenes spp. (Mundt & Hinkle, 1976), Arthrobacter globiformis (Ercolani, 1978), Bacillus (Ercolani, 1978), Brevibacterium (Mundt & Hinkle, 1976), Chromobacterium (Dickinson et al., 1975), Cor. (Clavibacter) michiganese (Mundt & Hinkle, 1976), Erwinia herbicola (Goodfellow et al., 1976; Ercolani, 1978), Lactobacillus (Ercolani, 1978), Pseudomonas fluorescens (Austin et al., 1978) and Serratia as Ser. marcescens (Ercolani, 1978) have been documented. Although it is difficult to compare numerical phenetic analyses with those based upon a few tests, it should be noted that Bacillus (Haikara et al., 1977; Douglas & Flannigan, 1988; Pepper & Kiesling, 1963), Pseudomonas and Erwinia herbicola (Haikara et al., 1977) and Lactobacillus (Sheneman & Hollenbeck, 1960) have been previously associated with the malting process, notably in dried barley kernels and finished malt. In addition, Brevibacterium linens,

regarded as a rare species on barley (Pepper & Kiesling, 1963), was detected in this study as a minor group. However, in contrast to other work, there was a total absence of Escherichia coli and Micrococcus (Haikara et al., 1977) and Xanthomonas campestris (Clarke & Hill, 1981; Flannigan et al., 1982) in this study. Instead, sizeable populations of coryneforms were recovered throughout the various stages of production. These organisms were difficult to identify, as was experienced by earlier workers, e.g. Austin et al. (1978). The taxonomic dilemma of the coryneforms is illustrated in the reclassification of 'Corynebacterium betae' and 'Cor. poinsettiae' as Curtobacterium flaccumfaciens subsp. betae and Curtobacterium flaccumfaciens subsp. poinsettiae respectively (Collins & Jones, 1983).

Although there is no previous record of Flav. esteroaromaticum associated with malting barley, it is known that this organism is a common inhabitant of aquatic environments (Thurner & Busse, 1978; Lee et al., 1981; Rheinheimer, 1976). This may likely be the source of contamination of the malting barley. Apart from Lactobacillus spp., E. herbicola, Ps. fluorescens and B. linens (Pepper & Kiesling, 1963), the remaining groups are mentioned here for the first time in association with barley or malt.

Alcaligenes sp. was restricted to only the steeped barley and water in this study. Besides the association with plant materials (Mundt & Hinkle, 1976), Alcaligenes is known to occur in water, soil and the intestinal tract of vertebrates (Kersters & De Ley, 1984). This may suggest that its presence in the malting process is from water.

Failure to detect either Cla. iranicum or Cla. michiganese in the water is not surprising because these organisms are known to be plant

pathogens that survive between crops in or within seed (Richardson, 1979), and their survival in soil is extremely limited.

The identification of Arthrobacter globiformis was aided by the clustering of the reference strain in this phenon. It was interesting to note its complete absence from dried barley to steeped out grain, but being detected in green, kilned and screened malts. It appears to be one of those organisms that is being stimulated to multiply during kilning. The same observation was noted in the case of Clavibacter iranicum. Isolation of Arthrobacter from malting barley was not surprising, because a large number of studies has shown that soil is the usual habitat of authentic members of the genus Arthrobacter. Thus, arthrobacters have commonly been shown to form a numerically important fraction of the indigenous flora of soils from various parts of the world, and may be the most numerous bacterial group encountered in aerobic total plate counts (e.g. Mulder & Antheunisse, 1963; Skyring & Quadling, 1969; Holm & Jensen, 1972; Hagedorn & Holt, 1975). However, arthrobacters become less numerous with increasing soil acidity (Lowe & Gary, 1972; Hagedorn & Holt, 1975).

The ubiquitous nature of E. herbicola may likely suggest why this organism is found at all stages of malting (Haikara et al., 1977), as was the case in this study. This organism is present in diseased areas on plants, on plant surfaces, in man and animals, and, occasionally, in soil, water and air (Lelliot & Dickey, 1984).

The presence of pseudomonads on barley (Pepper & Kiesling, 1963) and on all sample types of malting barley had previously been reported (Haikara et al., 1977). In contrast to the observation made by Haikara et al. (1977), the populations of Ps. putida (phena 24 and 25) were restricted to the first steeped barley corns (phenon 24) and green malt

(phenon 25). However, Haikara et al. (1977) did not specify which particular species of Pseudomonas was found in the sample types examined in their study. Although pseudomonads are regarded as part of the normal microflora on plants (Austin et al., 1978), they are also associated with spoilage of food such as eggs, cured meat, fish and milk (Palleroni, 1984).

The fact that lactobacilli are contaminants in the fermentation industry (Rainbow, 1979; Priest, 1987), is of considerable interest and importance. Lactobacillus spp. have been noted to contribute to the flavour of fermented food by producing various volatile compounds, such as diacetyl, its derivatives, H_2S and amines in cheese (Sharpe & Franklin, 1962; Law & Kolstad, 1983). Although the recovery of mesophilic lactobacilli agrees with the data published by Sheneman & Hollenbeck (1960), it differs in that L. leishmanii (which they identified) was not isolated in the present study. However, their finding that the highest population of lactic acid bacteria occurred during steeping is in good accord with the present study. Furthermore, these authors found thermophilic lactic acid bacteria to be extremely low in number, and lacking a definite growth pattern during the malting process. They suggested that these organisms might probably be present only as sporadic or incidental contaminants. The same might be said about organisms such as Aureobacterium flavescens, Oerskovia turbata, Clavibacter michiganese, Serratia plymuthica, Ser. rubidaea and Pseudomonas putida (phena 24 & 25).

The range of filamentous fungi recovered in this study is in line with previous observations of the mycoflora of plant organs and barley malt. Thus, representatives of the genera Alternaria, Aspergillus, Cladosporium, Fusarium, Mucor, Penicillium and Phoma have been

recovered from healthy leaves of a wide variety of plant species (Lamb & Brown, 1970; Ruscoe, 1971; Bewley, 1979) and barley kernels (Pepper & Kiesling, 1963; Gyllang & Martinson, 1976; Flannigan et al., 1982; Haikara et al., 1977; Douglas & Flannigan, 1988). Consistently, Alternaria alternata has been regarded as the most common field fungus on malting barley and as being a prominent species during malting (e.g. Haikara et al., 1977). Yet although Haikara et al. (1977) observed an increase in the percentage frequency of kernels bearing Alt. alternata, during laboratory steeping, Gyllang & Martinson (1976b) noted a decline during steeping, germination and kilning in the course of production of a commercial malt. Epicoccum has been isolated previously from malting barley (Flannigan et al., 1982), and Geotrichum candidum from green malt (Douglas & Flannigan, 1988). Indeed, G. candidum may become one of the dominant filamentous fungi on green malt, and is found on the kilned product (Healy, 1985). This study supports the general contention that G. candidum occurs only rarely in dry barley kernels (Pepper & Kiesling, 1963). Nevertheless, there were marked differences from other studies. Douglas & Flannigan (1988) reported the widespread occurrence of Cladosporium herbarum and C. cladosporioides and, at a lesser frequency, Aureobasidium pullulans on green malt. Neither Aureobasidium nor Cladosporium was prominent, however, in the present study, although Aspergillus glaucus, which was commonly recovered after germination and on kilned malt by Douglas & Flannigan (1988), was especially prevalent. Yet, these species were not found on green malt. Although Mucor has also been found to increase markedly during kilning (Haikara et al., 1977; Douglas & Flannigan, 1988), this was not observed in the present study. This may be attributed to a low level occurrence, i.e. beyond the level of detection.

Although nearly all kernels of screened malt (Table 10) were contaminated by Asp. glaucus group, the numbers populated by other categories of fungi were all lower than those in the original barley, except in the case of Absidia corymbifera and G. candidum. However, the MFI for the field fungi was higher on dried barley (1.37) than on screened malt (0.7). This phenomenon was mainly due to the high percentage contamination by Alt. alternata (46%) on dried barley. In contrast, the MFI for storage fungi was higher on the screened malt (1.56) than on the dried barley. This high MFI was a result of high percentage contamination of kernels by Asp. glaucus (98%) (Table 10).

Although a comparatively small fraction of the total yeast counts determined on barley samples during malting were identified a total of seven genera and eight species were characterized (Table 11). These included Candida, Rhodotorula and Sporobolomyces, which are all common on leaf surfaces (Last & Warren, 1972; Bewley, 1979) and in barley malt (Flannigan et al., 1982). Hill & Lacey (1983a) noted the dominance on barley of Rhodotorula and Sporobolomyces, but such a trend was not apparent in the present study. However, considering the entire malting process, these two species were most frequently isolated (Table 11). In addition to these three organisms, Hansenula sp. has also been associated with barley (Pepper & Kiesling, 1963) as rare species. Indeed, the only isolate of this species was recovered in steep-water. The other genera included Kloeckera, Debaryomyces and Trichosporon. Whereas Trichosporon has been isolated from dried barley (Douglas & Flannigan, 1988) it was recovered from kilned malt in this study. Debaryomyces and Kloeckera are of particular interest because of their significance in brewing. Brady (1958) isolated one strain of D. hansenii from pitching yeasts. As isolated by Wiles (1950) from turbid

beers, Kl. brevis grew rapidly in beer, producing haze. It is noteworthy that 'killer' yeast strains occur most frequently within the genus Hansenula (Rainbow, 1981).

Influence of Selected Micro-organisms on Barley and Malt Analysis

With the realization that the malting process provides nutrients and an environment adequate for microbial development, it is not surprising for any added micro-organisms during malting to increase in numbers. Sheneman & Hollenbeck (1960) showed that large populations of various groups of bacteria were present on barley, and were capable of increasing by a factor of 700 during malting of the grain. Kotheimer & Christensen (1961) reported that bacteria and yeasts increased greatly under similar conditions. However, the multiplication of micro-organisms may be limited to those that can grow under the conditions employed during malting. It means that there would be some interactions established between the barley (host) and the micro-organism (pathogen) which may eventually affect the barley kernel characteristics. The effect of Fusarium spp. upon the characteristics of malt and beer were examined by Sloey & Prentice (1962). In the present study the effects of F. nivale and G. candidum on barley and malt characteristics were examined and the results seemed to, in general, vary slightly.

The barley inculated with F. nivale during malting Run I showed a poor growth of the inoculated species. It can be suggested that this poor growth of F. nivale was due to interactions with other micro-organisms in the environment, or that the malting conditions were not adequate for its growth. For example, Limonard (1968) observed that the recorded frequency of F. nivale on wheat was affected by the degree

to which Epicoccum was eliminated by hypochlorite disinfection. Although F. nivale was recovered from more sample types (Tables 15b and 16b, and Appendices 10a and 11a) in Run II than in Run I, its growth response was still poor. In addition, the proportion of kernels contaminated with this fungus was only 2% on screened malt. However, the effect of kilning and screening may have contributed to the low count on screened malt (Douglas & Flannigan, 1988).

In contrast, the applied mould G. candidum showed a marked proliferation at all stages of malting in the two Runs and was virtually the only organism that grew on dilution plates. Thus, this may suggest its ability to suppress the growth of other fungi by perhaps producing toxic substances. The viable counts of the mould showed the greatest increase during germination. This may be important when considering the observations of Gjertsen et al. (1963, 1965) who maintained that the growth of previously applied micro-organisms during malting, and particularly the germination period, was the cause of observed modifications in the characteristics of finished malts and beers. Compared with the control, the population of G. candidum on the inoculated malt was higher than on the controls in both Runs. Although the growth in the control followed the pattern described by Douglas & Flannigan (1988), i.e. rare on the dried barley in Run I, the situation was surprisingly different in Run II, because this organism was detected in dried barley as well as in other sample types. This may suggest the presence of G. candidum on dried barley as dormant propagules. Again, all sample types from the inoculated barley showed evidence of the presence of G. candidum.

The physical and chemical characteristics of barley or malt may be altered due to the growth of these micro-organisms. However, the

extent to which the alteration could go depends on many factors, some of which may include the duration of contact between the host and the pathogen and method of application of the pathogen. In this study, the micro-organisms were applied externally, thus limiting their penetration into the pericarp testa or below the husk. Again, the 7-8 day malting period might not be long enough for effective colonization and perhaps modification of the barley kernel. Nonetheless, some interesting observations were made during the study.

Despite the enormous microbial populations applied in the steep water there appeared to be no appreciable effect on the water uptake by the barley when compared with the controls (Tables 17a, b). However, as would be expected, there was a rapid increase of water uptake at the first steep (Briggs et al., 1981) in both categories of malts.

The percentage recovery of malt from barley depends on the gross physical and chemical changes occurring during malting. These are the results of degradation of reserve substances, the interconversion of materials in the living tissues and the flux of materials to the embryo and their incorporation into the living tissue, and the synthesis of new materials (Briggs et al., 1981). The leaching of dry matter from the barley during steeping (steep loss), oxidation to CO_2 and water during respiration (respiration loss) and separation of rootlets and culms during screening (root loss) are all contributing to the percentage recovery of malt (Briggs et al., 1981). The general trends observed by Sloey & Prentice (1962) were toward increases in steep and respiration loss, decreases in rootlet loss, and decreases in malt recovery from barley. This agrees with the present work as far as G. candidum inoculated malts are concerned (Tables 18a, b). In contrast, F. nivale inoculated malt showed increases in root loss and decreases

in steep and respiration loss, with decreases in malt recovery in malting Run I and an increase in malting Run II. However, the overall observation indicated that there were no appreciable differences between the inoculated and the control malts.

During germination, acrospire growth is commonly used as a rough guide to the progress of malting. When it has grown to approximately 0.75 - 1.0x the length of the barley kernel, modification is deemed to have progressed sufficiently for kilning to take place (Briggs et al., 1981). In general, the changes in growth-index (a measure of acrospire growth) varied slightly between inoculated and control malts (Table 19), but there was a striking decrease noted in F. nivale inoculated malt in the two Runs. This appeared to coincide with the observation of other workers, e.g. Sloey & Prentice (1962). These workers noted that F. nivale has a general inhibitory effect in malting, because rootlet growth and acrospire development were decreased.

Application of micro-organisms during malting also brings about chemical changes in malt characteristics (Prentice & Sloey, 1960; Sloey & Prentice, 1962). This group reported that the micro-organisms affecting the α-amylase level were representatives of the genus Fusarium. They further noted that a culture of Fusarium caused an increase in diastatic power. In the present study, F. nivale was found to significantly decrease diastatic power of the malt wort in malting Run I, but in the second Run there appeared to be no difference when compared with the controls. This indicates that further work needs to be carried out before firm conclusions may be drawn. However, the inconsistency in the results obtained with diastatic power was also observed by other workers (Sloey & Prentice, 1962). The reason for these discrepancies is uncertain, although it has been suggested that a

change in characteristics of the fungi during storage may be responsible (Sloey & Prentice, 1962). G. candidum inoculated malt wort showed decreases in diastatic power in both Runs. This was in good accord with the pattern described by Sloey & Prentice (1962), who noted that several micro-organisms, other than Fusarium, appeared to decrease α-amylase activity. Yet subsequent experiments with the same organism did not generate confirmatory results (Sloey & Prentice, 1962).

Increases in wort nitrogen might be viewed with concern, because high nitrogen levels are often associated with poor keeping quality of beer. According to Sloey & Prentice (1962), the origin of the enzymes responsible for proteolysis in malt is not known, but possible sources were suggested. Firstly, it was suggested that the protease of the applied micro-organisms may have given rise to increased protein modification. Secondly, growth of other micro-organisms during malting may have contributed. Thirdly, stimulation of the synthesis of barley proteases or release from a bound form by the action of the applied fungi may have occurred. As an example, gibberellic acid has been shown to increase soluble nitrogen in malts (Bawden et al., 1959; Dickson, 1960; Fleming & Johnson, 1961) and also starch degradation by increasing α-amylase and diastatic power (Fleming et al., 1962). Contrary to these observation, there was no significant difference in the soluble and total nitrogen levels between the inoculated and control malt worts (Tables 20a, b), but the values were all lower in the test samples than in the controls. However, the α-amino nitrogen levels were significantly lower in the test samples than those of the controls. In contrast, Asp. fumigatus and R. oryzae have been found to increase α-amino nitrogn and soluble nitrogen in beer (Gyllang et al., 1977).

Further evidence that micro-organisms applied to barley during malting may affect malt characteristics was provided by the examination of C.W.E. According to Briggs (1978), Pollock (1962) and Harns (1962), the changes occurring in the modification of barley are mediated by numerous hydrolytic enzymes, many of which increase greatly in amount during malting. The soluble compounds produced by the activity of these enzymes, e.g. amino acids and sugars, tend to accumulate in the grain (particularly in the endosperm) faster than they are utilized by the embryo. These constitute the C.W.E. of the grain (Briggs et al., 1981). On the other hand, under standard production conditions, fermentability of unboiled wort is inversely related to the levels of C.W.E. and of soluble nitrogen (Bathgate et al., 1978). Although no significant differences in the levels of C.W.E. existed in any of the samples between the tests and the controls, the values were all slightly lower in the test samples than in the controls, except in F. nivale inoculated malt in Run II, which had a slightly higher C.W.E. value. It has been reported that F. nivale causes a small decrease in extract yield (Sloey & Prentice, 1962).

H.W.E. is another parameter which may be affected by micro-organisms during malting. This represents all materials brought into solution in a sweet wort prepared from malt (Briggs et al., 1981). Compared with the controls, although there was no significant difference in the levels of H.W.E. (coarse-grind) in Run I, there seemed to be significant differences in fine-grind extracts. But the coarse-fine difference which is regarded as the index of carbohydrate modification indicated that the control malts were better modified than the inoculated ones in all sample types in Run I. In Run II, a significant difference was observed in the levels of H.W.E. for both

coarse and fine-grind, but only for G. candidum inoculated malts. Despite this, no appreciable difference existed between the coarse-fine difference. The observation that virtually all values for the test extracts were lower than those of the controls could be taken to suggest that the applied micro-organisms had some effect on the malt enzymes. Indeed this may be deduced from their effects on DP (Tables 20a, b).

Wort prepared from the F. nivale inoculated malts indicated higher wort colour values than the corresponding controls (Tables 20a, b), which might have been due to the pigment associated with the applied mycelium (brown-pink) (Sloey & Prentice, 1962). Kneen (1963) noted that malt prepared from weathered barley produced beer with a high colour, as was the case when malt prepared after inoculation with Asp. niger, Cephalothecium sp., Rhizopus sp. and especially Fusarium sp. or R. arrhizus was used. Increased colour has subsequently been observed when Asp. fumigatus, R. oryzae (Gyllang et al., 1977), F. avenaceum and F. culmorum (Haikara, 1983) have been present. In contrast, wort colour values from G. candidum inoculated malt were lower than those of the controls. However, all values of the wort colour for both F. nivale and G. candidum inoculated malts did not indicate significant differences when compared with the corresponding controls.

Percentage fermentability is an index of the distilling quality of the malt and a measure of the fermentable sugars present in the wort (Bathgate et al., 1978), and the same method was proposed for use in the brewing industry (Bathgate, 1981). There were very slight differences between the percentage fermentability values in worts prepared from the inoculated malts and those of the controls, except in F. nivale inoculated malt wort in Run I. Although the controls

appeared to be better modified than the inoculated malt, the low values in the percentage fermentability may be as a result of removal of potentially fermentable reducing sugars by condensation with amino acids (Bathgate et al., 1978), since the control malt had much greater value for total soluble nitrogen and total nitrogen ratio (index of protein modification). Alternatively, the inoculated malt might have had added hydrolytic enzymes from the applied micro-organisms; but this is not likely to be the case since the D.P. indicated lower levels in the inoculated malts.

While a distiller is interested in the maximization of alcohol production, a brewer places more emphasis in the total malt extract (Bathgate et al., 1978). However, in both categories alcohol is produced during fermentation, which is just one of the organoleptic compounds. Although there was little or no difference in the ethanol yields from the boiled wort prepared from the inoculated and the control malts, other defects such as off-flavour and mycotoxin effects may be encountered. For example, trichothecenes have been reported to retard yeast growth (Schappert & Khachatourians, 1983, 1984) and fermentation (Flannigan, 1986). Beer brewed with malt contaminated with R. oryzae was noted by Gyllang et al. (1977) as being distinctive, without forming off-flavour, but Asp. fumigatus gave a pronounced staling flavour. Although these parameters were not monitored in the present study, future work may include them.

Effects of Extracellular Enzymes on Some Carbohydrates Associated with Barley or Malt

Henry (1988) reviewed the carbohydrate composition of barley grains. Among the carbohydrates, β-glucans (Anderson et al., 1978),

pentosans (Henry, 1986), raffinose, sucrose and glucose (Macleod, 1952) were those of interest in this study. The ability of micro-organisms to degrade polysaccharides, such as glucan, starch, and cellulose is a characteristic of considerable interest both in terms of microbial ecology and from the viewpoint of industrial microbiology, in particular the malting, brewing and distilling industries. For example, Bamforth (1982) noted that added microbial β-glucanases caused improved degradation of both gums and hemicelluloses, with concomitant improvements in wort separation rates and yield of extract, together with the relief of problems at later stages in processing and storage. Hence, it would be of greater interest if micro-organisms indigenous to the malting environment or brewing process would produce extracellular enzymes capable of degrading especially the problem polysaccharides, e.g. barley β-glucan. In the present study, culture filtrates of some of the micro-organisms isolated during the commercial malting of barley were found to degrade barley β-glucan, starch, arabinoxylan (Tables 21a, b), sucrose and raffinose (Table 32). These results agree with the observations of previous workers insofar as microbial degradation of polysaccharides is concerned, though their sources of substrate and enzymes may be diffrent. Some bacteria and fungi capable of degrading cellulolytic compounds have been reported by Sazci et al., 1976; Teather & Wood, 1982; Mahasneh & Stewart, 1980; Carder, 1985; Wood & Weisz, 1987). Furthermore, enzyme systems capable of specifically hydrolysing 1,3-β-glucosidic linkages in glucans have been reported from fungi (Reese & Mandels, 1959) and from algae (Duncan et al., 1956; Felling, 1960). However, no attempt was made to differentiate which types of glucosidic linkages are hydrolysed in this study.

Under the conditions of study, (i.e. on plates) the numbers of

filamentous fungi capable of producing extracellular enzyme (β-glucanase) and degrading barley β-glucan (40° C) were more (41% of the total moulds) than either bacteria (28% of the total bacteria) or yeasts (25% of total yeasts). Although Trichoderma was not isolated in this study, it has been noted that its enzyme system is particularly efficient in degrading β-glucan (Stentebjerg-Olesen, 1980). Here, F. nivale was detected as showing high glucanase activity (Fig. 8) when compared with other isolates. The endo-β-glucanase produced by bacteria of the genus Bacillus has been reported to hydrolyse the mixed-linkage of β-glucans (Anderson et al., 1978). Specifically, Bacillus circulans (strain 1C9-12) isolated from garden soil was noted to be active in producing several glucanases including 1-3-β-glucanase (Mahasneh & Stewart, 1980). Such observations agree with the results in the present study which also implicated Bacillus spp. as producers of β-glucanase. In contrast, however, these species did not demonstrate great activity, except Bacillus sp. W12 which ironically was not isolated from malting barley. Other bacteria that also showed less activity were Flavobacterium esteroaromaticum, Clavibacter iranicum, Microbacterium imperiale, M. lacticum and Arthrobacter globiformis. Although the present investigation did not extend to studying degradation of carbohydrates under anaerobic conditions, other workers, for example Teather & Wood (1982), reported that rumen bacteria, including particular strains of Butyrivibrio fibrisolvens, Clostridium cellobioparum, and Ruminococcus flavefaciens, hydrolysed oat β-D-glucan. Already in use in the brewing industry are the β-glucanases of Bacillus subtilis and Asp. niger to degrade β-glucan (Stentebjerg-Olesen, 1980) but these are commercially produced.

Most of the yeasts tested did not indicate any hydrolysis of the

β-glucan in the medium and the few that seemed to react did not give hydrolysis to any great extent (Table 21b). The same pattern of reaction was observed when yeast isolates were tested for starch hydrolysis. Furthermore, these yeasts did not show any detectable hydrolysis when xylan compound was included in the medium. Since the optimal conditions were not determined for these organisms, failure to produce or give positive responses does not necessarily mean that they do not have the potential to hydrolyse the carbohydrate. However, there are some yeasts that are unable to assimilate a wide variety of carbon compounds. For instance, species of Cryptococcus, Rhodotorula, Candida, and Torulopsis are unable to assimilate a wide variety of carbon compounds (Hough et al., 1982).

More than 50% of the filamentous fungi tested in this survey were found to show xylanase activity. Arabinoxylan is a pentosan which, like β-glucan, is known to be present in viscous worts causing slow rates of run-off from the mash tun and filtration of the beer (Scott, 1972; Bathgate & Dalgleish, 1975). In addition, Preece & Hobkirk (1955a, b) pointed out that the activity of the pentosanase system of raw barley is very low. Hence the knowledge of micro-organisms that are able to degrade xylan will be of great help to breweries and distilleries. Flannigan (1970b) reported that culture filtrates of some fungal isolates from barley kernels degraded arabinoxylan, with the greatest activity being shown by Botrytis cinerea. Xylanase activity was observed less frequently among the bacterial isolates (3%) in this study.

Again without determining the optimum conditions for amylase activity for the individual micro-organisms, the degradative ability of the isolates on soluble starch was examined. The data suggested

that filamentous fungi and bacteria might make a great impact in the degradation of starch during malting, with filamentous fungi showing numerical dominance (Tables 21a, b).

Solution studies with barley β-glucan have shown that the microbial isolates have a characteristic range of optimal conditions for the production of β-glucanases. The bacteria developed the highest β-glucanase activity under the conditions of investigation, within 48 h, while the fungal species were noted to reach the peak on the fifth day of incubation. However, the fungal species tended to produce more β-glucanase than the bacterial isolates. For example, measured in terms of β-glucanase activity, F. nivale (at five days) gave 13.0 μg ml^{-1} glucose, while Bacillus sp. (phenon 6) showed only 9.3 μg ml^{-1} glucose. In contrast, not all the organisms tested showed positive responses, nor appreciable activity. G. candidum, for instance, consistently demonstrated a non-reactive response in all tests (Tables 22-24). This is quite different from the reports given by Vasil'eva et al. (1987) that G. candidum 3C was found to produce at least two endoglucanases, two cellobiases, and an enzyme splitting the cotton fibre cellulose into sugar, one galactanase, one mannase, two arabinases, one endoxylanase, two β-D-galactosidases and two β-D-glucosidases. This may probably be attributed to differences among strains. Arthrobacter globiformis showed low glucanase activity (1.2 μg ml^{-1} glucose). The low level of β-glucanase production by this organism may be a result of its slow growth rate. Thus, members of this genus are characteriz ed by slow growth (Stevenson, 1967).

On the basis of the results present (Fig. 9 & Table 23), it would appear that at least two peaks of pH values are apparent for the bacteria tested (pH 5.6 and 7.0) for glucanolysis, with higher activity

of the latter. The fungal cultures of F. nivale and F. avenaceum showed maximum activity at pH 6.6.

Viscometric studies revealed that culture filtrates of those bacterial cultures that showed β-glucanase activity on plates and even in liquid media had no effect on the viscosity of the β-glucan substrate. This suggests that the enzyme was mostly exo-β-glucanase. However, it has been found that immobilization of endo-β-glucanases can change their mode of action to an exo-form, in which case they are much less effective in reducing viscosity (Svesson & Otteson, 1978). Again, Zaikina et al. (1985) noted that exo-1,3-β-glucanases from G. candidum 3C are highly specific and would fail to degrade mixed types of bonds in barley glucan. The culture filtrate of F. nivale was the only such prepartion that mediated an appreciable reduction in viscosity (Fig. 10). Approximately 19.2 units of enzymes were required to bring about a 10% reduction in viscosity of the β-glucan substrate in 100 min per assay. However, even though there appears to be an appreciable reduction in viscosity, it must be remembered that the substrate, i.e. β-glucan, was pure; the situation may be different in an industrial context, e.g. in wort separation, and this must be considered in any future study.

The studies on the effect of temperature on the growth of, and extracellular enzyme production in, F. nivale and G. candidum revealed that given suitable nutrients F. nivale grew over the temperature range of 4-25° C (Table 25), growth at the lower end of this range being in line with its common name, "snow-mold" (Tindall, 1986). However, G. candidum only grew well over the temperature range 18-30° C (Table 26). Although F. nivale was observed to grow at temperatures below 18° C, it is interesting that its ability to produce β-glucanase appeared to be

limited to a temperature range 18-25° C, whilst it produced amylases over the range 10-25° C. Nevertheless, since malting and brewing temperatures for production in commercial runs are within these ranges, the enzymes from F. nivale will have important implications in degrading the β-glucan or starch of the barley or adjunct. In particular, the temperature range during germination of barley in malting is 12 - 18° C, and for fermentation, 10 - 16° C depending on the type of yeast used. G. candidum, in contrast, did not show any noticeable β-glucanase activity nor amylolytic activity at the various temperatures examined.

Although the earlier experiments have indicated amylolytic activity in the culture filtrate of F. nivale, the work was extended in a limited way to determining the type of amylases that may be produced. However, no attempt was made to examine β-amylase. Since α-amylase degrades the glucosidic bonds of starch or β-limit dextrin randomly, the rapid reduction in starch/dextrin-iodine complex indicates α-amylase (endo-enzyme) activity. A gradual decrease would show the presence of exo-enzymes. From the relationship of amylolytic activities of F. nivale, measured by reduction in iodine staining power of soluble starch and that by reducing power (Fig. 14 & Table 31), it may be suggested that the culture filtrate contains mainly α-amylase. However, the typical curve expected from such a relationship was not obtained, suggesting that other enzymes other than α-amylase were present in the culture filtrate. Indeed, the culture filtrate was in itself a crude enzyme preparation. Hence a more specific substrate was used to confirm α-amylase presence. Since hydrolysis of β-limit dextrin is regarded as being specific to α-amylase it therefore means that the reduction of its iodine staining confirms α-amylase production

by F. nivale (Fig. a, b and Tables 29, 30). However, fungal α-amylases are reported to be unfortunately heat-labile (they are inactivated at 60° C) for use in mashing, although a high maltose-producing fungal α-amylase has been used to 'prime' beer and its heat lability is an advantage, since it is readily inactivated during pasteurization (Briggs et al., 1981). Nevertheless, since the temperature during malting does not rise that high before kilning, the fungal α-amylases will also find their use during the modification of barley, as has already been pointed out.

The culture filtrate of F. nivale was also found to degrade both raffinose and sucrose under the conditions of study, whereas culture filtrate from G. candidum did not. Since not all brewing yeasts can utilized raffinose efficiently, its degradation to simpler sugars is an advantage, e.g. Saccharomyces carlsbergensis uses raffinose completely, whereas S. cerevisiae only uses it partially (Hough et al., 1982).

Conclusions

It is apparent that a much greater diversity in bacterial groups occur in the stages involved in barley malt production than has hitherto been realised. In part, this may reflect inadequate approaches to taxonomy in the past, or indicate basic differences between laboratory based- and commercial malting. However, the role of these bacteria, and the filamentous fungi and yeasts in the malting process is far from clear. Indeed, from the micro-malting studies it is difficult to draw any firm conclusions because of the inconsistencies in the results of the two malting Runs. However, for the present, it is apparent that F. nivale grew poorly during the

malting process whereas G. candidum multiplied well in all samples throughout the malting process. Certainly, these organisms appeared to affect the physical and chemical characteristics of the finished malt, including root loss, steep and respiration loss and acrospire length, although they did not exert any substantial influences on most of the characters. Since the culture filtrate of F. nivale degraded β-glucan and caused a reduction in viscosity of the substrate, and further degraded starch, sucrose and raffinose (Contrasting with the apparent non-reactivity of G. candidum) its application to malt wort, a complex substrate could be considered. A study of the purified enzyme(s) could also have relevance for the industry.

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Appendix 1

Student's t-test on the numbers of micro-organisms associated with pairs of sample situations.

Sample pairs	Organisms	Level of significance	Remarks
Dry barley/ screened malt	bacteria	99.5%	highly significant
Second steep grain/ green malt	bacteria	75.0%	same
Second steep grain/ green malt	presumptive lactobacilli	100.0%	highly significant
Second steep grain screened malt	presumptive lactobacilli	99.0%	highly significant
Kilned malt/ screened malt	filamentous fungi	99.7%	highly significant
Dried barley/ first steep grain	filamentous fungi	99.7%	highly significant
Dried barley/ screened malt	filamentous fungi	99.8%	highly significant
Second steep grain/ green malt	yeasts	100.0%	highly significant
Kilned malt/ screened malt	yeasts	98.5%	highly significant

Appendix 2

Calculation and results of test error

Test Code (N)	n	V*	Test Code (N)	n	V
1	1	0.02	49	3	0.06
2	0	0.00	50	1	0.02
3	0	0.00	51	0	0.00
4	0	0.00	52	0	0.00
5	1	0.02	53	0	0.00
6	1	0.02	54	0	0.00
7	1	0.02	55	0	0.00
8	0	0.00	56	2	0.04
9	0	0.00	57	5	0.10
10	0	0.00	58	4	0.08
11	2	0.04	59	4	0.08
12	0	0.00	60	3	0.06
13	0	0.00	61	1	0.02
14	0	0.00	62	4	0.08
15	1	0.02	63	2	0.04
16	1	0.02	64	4	0.08
17	0	0.00	65	2	0.04
18	2	0.04	66	0	0.00
19	2	0.04	67	0	0.00
20	3	0.06	68	4	0.08
21	1	0.02	69	3	0.06
22	0	0.00	70	3	0.06
23	4	0.08	71	4	0.08
24	3	0.06	72	2	0.04
25	3	0.06	73	2	0.04
26	4	0.08	74	4	0.08
27	1	0.02	75	4	0.08
28	1	0.02	76	4	0.08
29	4	0.08	77	3	0.06
30	0	0.00	78	1	0.02
31	0	0.00	79	4	0.08
32	4	0.08	80	0	0.00
33	1	0.02	81	3	0.06
34	4	0.08	82	2	0.04
35	3	0.06	83	0	0.00
36	1	0.02	84	1	0.02
37	0	0.00	85	1	0.02
38	0	0.00	86	1	0.02
39	4	0.08	87	3	0.06
40	2	0.04	88	1	0.02
41	1	0.02	89	3	0.06
42	0	0.00	90	4	0.08
43	0	0.00	91	2	0.04
44	4	0.08	92	0	0.00
45	4	0.08	93	2	0.04
46	2	0.04	94	1	0.02
47	1	0.02	95	3	0.06
48	1	0.02	96	2	0.04

Appendix 2 (cont.)

Test Code (N)	n	V*
97	2	0.04
98	1	0.02
99	4	0.08
100	4	0.08
101	4	0.08
102	4	0.08
103	4	0.08
104	3	0.06
105	4	0.08
106	4	0.08
		3.92

*Variance (V) = S_i^2 = n/2t, where n = no. of OTU's with discrepancies;
t = 25 (total no. of strains)

$S = \not\Sigma V/N = 3.92/106 = 0.037$

The probability (p) of an erroneous result is given by the equation:

$$p = 1/2[1-\sqrt{(1-4 \times S)}]$$

$$= 1/2[1-\sqrt{(1-4 \times 0.037)}]$$

$$= 1/2[1-\sqrt{(1-0.148)}]$$

$$= 1/2[1-\sqrt{0.852}]$$

$$= 1/2(1-0.923) = 1/2 \times 0.077 = 0.038 = \underline{3.8\%}$$

Appendix 3

Characteristics of the borehole water bacterial isolates

No. of Strains	Mucoid	Gram reaction	Catalase test	Oxidase test	Pigmentation	Cell shape
30	+	-	+	+	C	R
5	+	+	+	+	C	R
2	+	-	+	-	C	R
1	+	-	-	+	C	R
5	+	+	-	-	Y	R
2	+	-	+	+	Y	R
1	+	-	+	-	Y	R
1	+	V	-	-	Y	R
1	+	+	+	+	Y	R

V = variable; C = cream/white pigment, Y = yellow pigment; R = rods.

Appendix 4a

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of control malt in malting Run I; and total viable counts of all micro-organisms.

CFu5	Dried barley VU K^{-1}	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Field fungi								
Fusarium nivale	-	-	-	-	-	-	10	-
Fusarium spp.	-	-	40	$1.7x10^4$	-	-	-	-
Geotrichum candidum	-	-	-	-	$3.7x10^2$	70	-	-
Storage fungi								
Aspergillus candidus	$9.7x10^2$	-	-	-	-	-	-	-
Aspergillus glaucus	$1.7x10^2$	-	10	10	$1.0x10^2$	-	$4.7x10^2$	$5.7x10^2$
Penicillium spp.	$1.1x10^3$	$1.3x10^2$	$1.0x10^2$	$1.0x10^3$	$4.7x10^2$	$1.0x10^2$	$1.0x10^2$	70
Total viable counts								
Total moulds	$2.2x10^3$	$1.3x10^2$	$1.0x10^2$	$1.8x10^4$	$9.4x10^2$	$1.7x10^2$	$5.8x10^2$	$6.4x10^2$
Total yeasts	$1.7x10^3$	$2.7x10^2$	$3.0x10^2$	$1.5x10^5$	$1.0x10^4$	$1.6x10^4$	$8.6x10^3$	$1.8x10^4$
Total lactic acid bacteria	$7.3x10^2$	$3.7x10^3$	$1.0x10^3$	$1.3x10^6$	$7.6x10^4$	$1.7x10^5$	$3.5x10^3$	$9.5x10^5$
Total bacteria	$6.9x10^4$	$9.0x10^4$	$8.5x10^5$	$9.1x10^6$	$3.9x10^5$	$4.8x10^6$	$4.2x10^5$	$5.6x10^6$

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 4b

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of control malt in malting Run I; and total viable counts of all micro-organisms.

CFm7	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Field fungi							
Geotrichum candidum	-	-	$1.3x10^4$	$1.0x10^2$	$2.7x10^2$	-	-
Mucor spp.	-	-	-	$1.7x10^2$	$1.0x10^2$	-	-
Storage fungi							
Aspergillus candidus	30	$5.7x10^2$	$2.3x10^2$	70	70	$8.3x10^2$	$4.7x10^2$
Aspergillus flavus	70	$1.7x10^2$	-	-	-	-	70
Aspergillus glaucus	-	$5.7x10^2$	-	-	70	$1.3x10^2$	$1.3x10^2$
Penicillium ssp.	-	$1.3x10^2$	-	-	-	$1.0x10^2$	70
Total viable counts							
Total moulds	$1.0x10^2$	$1.4x10^3$	$1.3x10^4$	$3.4x10^2$	$5.1x10^2$	$1.0x10^3$	$1.4x10^3$
Total yeasts	$1.9x10^3$	$4.9x10^3$	$3.3x10^5$	$6.1x10^3$	$2.6x10^3$	$1.9x10^3$	$2.1x10^4$
Total lactic acid bacteria	$9.0x10^2$	$3.1x10^3$	$6.5x10^6$	$1.4x10^5$	$9.6x10^4$	$9.3x10^2$	$3.4x10^5$
Total bacteria	$1.5x10^6$	$4.6x10^7$	$1.5x10^8$	$1.2x10^6$	$7.8x10^6$	$3.3x10^5$	$1.2x10^7$

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 5a

Moulds detected during micro-malting of control I in malting Run I by direct plating, percentage contamination by different moulds, and MFI's.

CFm5	Dried barley	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination					
Field fungi						
Alternaria alternate	24	-	-	-	-	-
Aureobasidium pullulans	-	-	-	-	3	2
Botrytis cinerea	12	-	-	-	-	-
Cochliobolus sativum	-	-	-	-	7	-
Epicoccum purpurascens	6	-	2	-	-	-
Fusarium nivale	-	-	29	-	-	-
Fusarium spp.	10	33	-	-	-	-
Geotrichum candidum	-	-	34	-	10	6
Mucor spp.	-	-	-	30	28	14
Storage fungi						
Aspergillus spp.	-	-	-	43	-	-
A. candidus	4	-	-	-	-	1
A. flavus	2	-	-	-	-	-
A. glaucus	100	100	100	-	100	98
Penicillium spp.	40	16	11	105	65	72
MFI's						
Field fungi	0.52	0.33	0.65	0.30	0.48	0.22
Storage fungi	1.46	1.16	1.11	1.48	1.65	1.71
All fungi*	1.98	1.49	1.76	1.78	2.13	1.93

- =Not detected.

Appendix 5b

Moulds detected during micro-malting of control II in malting Run I by direct plating, percentage contamination by different moulds, and MFI's.

CFM7	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination				
Field fungi					
Absidia spp.	2	-	-	-	-
Alternaria alternata	-	-	4	-	-
Aureobasidium pullulans	-	2	-	-	-
Botrytis cinerea	22	2	-	-	-
Epicoccum purpurascens	4	-	-	-	-
Fusarium spp.	6	4	-	-	-
F. nivale	-	4	-	-	-
G. candidum	16	22	86	16	34
Mucor spp.	20	2	28	54	34
Storage fungi					
Aspergillus spp.	2	-	-	-	-
A. candidus	4	-	8	4	6
A. glaucus	100	100	90	100	100
Penicillium spp.	18	24	32	4	38
MFI's					
Field fungi	0.72	0.36	1.18	0.70	0.64
Storage fungi	1.24	1.24	1.30	1.04	1.44
All fungi	1.96	1.60	2.48	1.74	2.08

- = Not detected.

Appendix 6a

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of control malt inoculated with Fusarium nivale in malting Run I; and total viable counts of all micro-organisms.

TF@5$_1$	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Field fungi							
Fusarium nivale	80	$1.0x10^2$	-	-	-	90	-
Fusarium spp.	-	40	-	-	-	-	-
Storage fungi							
Aspergillus candidus	-	-	-	-	-	$1.1x10^3$	-
Aspergillus glaucus	-	10	0	0	0	$2.3x10^2$	$2.0x10^2$
Penicillium spp.	$1.2x10^3$	$4.0x10^2$	$1.3x10^6$	$5.3x10^4$	$2.3x10^4$	$1.9x10^4$	$2.0x10^3$
Total viable counts							
Total moulds	$1.3x10^3$	$5.5x10^2$	$1.3x10^6$	$5.3x10^4$	$2.3x10^4$	$2.0x10^4$	$2.2x10^3$
Total yeasts	$1.0x10^2$	$3.0x10^2$	$5.0x10^5$	-	$4.0x10^3$	$8.3x10^3$	70
Total lactic acid bacteria	$4.0x10^3$	$5.0x10^3$	$1.4x10^5$	$3.1x10^4$	$1.0x10^5$	$3.0x10^3$	$4.8x10^4$
Total bacteria	$1.0x10^5$	$4.9x10^5$	$8.3x10^6$	$3.1x10^5$	$3.5x10^6$	$3.4x10^5$	$3.2x10^6$

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 6b

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of control malt inoculated with Geotrichum candidum in malting Run I; and total viable counts of all micro-organisms.

TFm7$_1$	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Field fungi							
Geotrichum candidum	2.1×10^5	8.3×10^3	2.3×10^6	2.4×10^5	5.8×10^5	4.6×10^5	7.5×10^6
Storage fungi							
Aspergillus candidus	30	1.2×10^3	6.3×10^2	3.7×10^2	20	5.3×10^2	1.7×10^2
Aspergillus glaucus	30	1.0×10^2	-	-	10	1.6×10^2	70
Penicillium spp.	-	-	-	-	-	70	70
Total viable counts							
Total moulds	2.1×10^5	9.6×10^3	2.3×10^6	2.4×10^5	5.8×10^5	4.6×10^5	7.5×10^6
Total yeasts	-	-	-	-	-	-	-
Total lactic acid bacteria	9.7×10^2	1.2×10^2	9.0×10^4	5.5×10^3	2.0×10^4	6.0×10^2	2.0×10^3
Total bacteria	4.5×10^5	3.1×10^5	6.8×10^5	6.6×10^5	1.2×10^6	3.0×10^5	7.6×10^6

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 7a

Moulds detected during micro-malting of malts inoculated with *Fusarium nivale* in malting Run I by direct plating, percentage contamination by different moulds and MFI's.

F. nivale	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination				
Field fungi					
Absidia spp.	3	-	100	51	40
F. nivale	12	-	-	-	-
Storage fungi					
Aspergillus glaucus	-	31	-	40	43
Penicillium spp.	100	100	100	99	100
MFI's					
Field fungi	0.15	-	1.00	0.51	0.40
Storage fungi	1.00	1.31	1.00	1.39	1.43
All fungi	1.15	1.31	2.00	1.90	1.83

- = Not detected.

Appendix 7b

Moulds detected during micro-malting of malts inoculated with *Geotrichum candidum* in malting Run I by direct plating, percentage contamination by different moulds; and MFI's.

G. candidum	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination				
Field fungi					
Geotrichum candidum	100	100	100	100	98
Mucor spp.	-	-	-	2	2
Storage fungi					
Aspergillus sp.	-	-	-	-	2
A. candidus	8	12	12	-	8
A. glaucus	74	94	94	100	96
Penicillium spp.	10	16	20	6	14
MFI's					
Field fungi	1.00	1.00	1.00	1.02	1.00
Storage fungi	0.92	1.22	1.26	1.06	1.20
All fungi	1.92	2.22	2.26	2.08	2.08

- = Not detected.

Appendix 8a

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of control malt in malting Run II; and total viable counts of all micro-organisms.

CFm5_2	Dried barley VU K^{-1}	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Storage fungi								
Aspergillus sp.	20	$1.3x10^2$	-	-	-	-	-	-
A. candidus	20	$3.3x10^2$	$1.3x10^2$	$2.0x10^2$	$1.0x10^2$	60	$8.7x10^2$	$1.3x10^2$
A. glaucus	$2.7x10^2$	$2.0x10^2$	$2.7x10^2$	30	-	70	$2.7x10^2$	$1.7x10^2$
Penicillium spp.	-	30	50	30	-	90	$1.5x10^3$	-
Total viable counts								
Total moulds	$2.9x10^2$	$6.9x10^2$	$4.5x10^2$	$2.6x10^2$	$1.0x10^2$	$2.2x10^2$	$2.6x10^3$	$3.0x10^2$
Total yeasts	40	$6.3x10^2$	$1.0x10^3$	$1.2x10^6$	$1.4x10^4$	$2.6x10^3$	$1.5x10^3$	$2.3x10^4$
Total lactic acid bacteria	40	$1.0x10^3$	$3.0x10^2$	$8.0x10^4$	$3.6x10^3$	$3.5x10^4$	$1.5x10^3$	$1.9x10^4$
Total bacteria	$6.1x10^3$	$2.8x10^5$	$2.4x10^6$	$9.1x10^7$	$9.6x10^6$	$1.0x10^7$	$4.0x10^5$	$3.8x10^6$

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 8b

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of control malt II in malting Run II; and total viable counts of all micro-organisms.

CFM7$_2$	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Field fungi							
Geotrichum candidum	-	-	$1.1x10^4$	$4.7x10^3$	$1.7x10^3$	-	-
Mucor spp.	-	-	-	$1.7x10^3$	$2.0x10^3$	-	-
Storage fungi							
Aspergillus candidus	$1.2x10^3$	$7.7x10^2$	$1.7x10^2$	-	80	$2.3x10^2$	$3.0x10^2$
Aspergillus glaucus	-	-	-	70	10	$1.0x10^2$	$1.0x10^2$
Penicillium spp.	-	-	30	-	-	$1.7x10^2$	$2.7x10^2$
Total viable counts							
Total moulds	$1.2x10^3$	$7.7x10^2$	$1.1x10^4$	$6.4x10^3$	$3.7x10^3$	$5.0x10^2$	$6.7x10^2$
Total yeasts	70	$7.3x10^2$	$1.3x10^4$	$2.7x10^3$	$3.8x10^3$	$7.7x10^2$	$3.7x10^3$
Total lactic acid bacteria	$2.3x10^3$	$2.6x10^5$	$2.1x10^5$	$2.0x10^3$	$1.0x10^4$	$1.7x10^3$	$2.0x10^4$
Total bacteria	$1.4x10^5$	$6.8x10^5$	$7.0x10^6$	$5.1x10^6$	$3.8x10^6$	$3.2x10^5$	$6.2x10^7$

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 9a

Moulds detected during micro-malting of controls in malting Run II by direct plating, percentage contamination; and MFI's.

CFm5	Dried barley	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination					
Field fungi						
Absidia spp.	1	-	-	-	-	-
Alternaria alternata	6	4	-	-	-	-
Aureobasidium pullulans	1	-	-	-	-	-
Fusarium spp.	28	2	-	-	8	2
Geotrichum candidum	4	12	28	32	6	76
Mucor spp.	-	10	-	16	16	10
Rhizopus spp.	5	-	-	-	-	-
Storage fungi						
Aspergillus candidum	3	2	2	2	4	4
A. flavus	2	2	-	-	-	-
A. glaucus	97	100	98	88	98	98
Penicillium spp.	32	42	8	52	24	10
MFI's						
Field fungi	0.45	0.26	0.28	0.48	0.30	0.88
Storage fungi	1.32	1.46	1.08	1.42	1.26	1.22
All fungi	1.75	1.74	1.36	1.90	1.56	2.10

- = Not detected.

Appendix 9b

Moulds detected during micro-malting of control II in malting Run II by direct plating, percentage contamination; and MFI's.

CFm7	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination				
Field fungi					
Aureobasidium pullulans	-	6	-	-	-
Epicoccum purpurascens	-	4	-	-	-
Fusarium spp.	2	4	-	-	-
Geotrichum candidum	60	48	100	92	12
Mucor spp.	-	-	-	2	72
Storage fungi					
Aspergillus candidus	2	8	6	2	-
A. glaucus	100	94	72	98	100
Penicillium spp.	30	8	44	-	4
MFI's					
Field fungi	0.62	0.62	1.00	0.94	0.84
Storage fungi	1.32	1.10	1.22	1.00	1.04
All fungi	1.94	1.72	2.22	1.94	1.88

- = Not detected.

Appendix 10a

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of malt inoculated with Fusarium nivale in malting Run II; and total viable counts of all micro-organisms.

TFm5₂	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Field fungi							
Fusarium nivale	$1.5x10^3$	$2.0x10^2$	$1.0x10^3$	-	-	$3.0x10^2$	-
Storage fungi							
Aspergillus candidus	30	$3.0x10^2$	$4.7x10^2$	-	-	$7.0x10^2$	$1.0x10^2$
Aspergillus glaucus	$2.3x10^2$	$1.0x10^2$	-	50	-	$1.7x10^2$	$1.7x10^2$
Penicillium spp.	$1.0x10^2$	-	-	-	-	-	-
Total viable counts							
Total moulds	$1.8x10^3$	$6.0x10^2$	$1.4x10^3$	50	-	$1.1x10^3$	$2.7x10^2$
Total yeasts	$2.7x10^2$	$2.4x10^3$	$3.0x10^5$	$6.8x10^3$	$6.3x10^3$	$8.0x10^2$	$1.0x10^4$
Total lactic acid bacteria	$1.0x10^3$	$2.0x10^2$	$7.8x10^4$	$5.2x10^3$	$1.0x10^4$	$9.3x10^2$	$1.5x10^4$
Total bacteria	$1.0x10^5$	$1.7x10^6$	$8.1x10^7$	$5.7x10^6$	$5.0x10^6$	$3.2x10^5$	$2.9x10^6$

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 10b

Viable counts of different moulds detected by dilution plating of samples taken during micro-malting of malt inoculated with Geotrichum candidum in malting Run II; and total viable counts of all micro-organisms.

TFm7₂	Barley after first steep VU K^{-1}	Steep out barley VU K^{-1}	Green malt VU K^{-1}	Kilned malt VU K^{-1}	Screened malt VU K^{-1}	First steep water VU ml^{-1}	Second steep water VU ml^{-1}
Field fungi							
Geotrichum candidum	$1.0x10^5$	$1.5x10^5$	$4.9x10^6$	$5.7x10^4$	$7.9x10^4$	$2.4x10^7$	$2.9x10^7$
Storage fungi							
Aspergillus candidus	$1.2x10^3$	$3.0x10^2$	$3.0x10^2$	-	$2.3x10^2$	$8.0x10^2$	$2.0x10^2$
Aspergillus glaucus	$2.7x10^2$	$1.7x10^2$	-	70	70	$3.0x10^2$	$2.3x10^2$
Penicillium spp.	-	-	$1.0x10^2$	-	-	-	$1.0x10^2$
Total viable counts							
Total moulds	$1.0x10^5$	$1.5x10^5$	$4.9x10^6$	$5.7x10^4$	$7.9x10^4$	$2.4x10^7$	$2.9x10^7$
Total yeasts	-	-	-	-	-	-	-
Total lactic acid bacteria	-	$4.1x10^3$	$1.9x10^3$	$9.3x10^2$	70	$5.6x10^3$	$2.2x10^3$
Total bacteria	$3.7x10^4$	$4.0x10^5$	$7.4x10^6$	$9.3x10^5$	$4.0x10^6$	$1.0x10^4$	$2.3x10^6$

- = Not detected.

VU K^{-1} denotes viable units per kernel

Appendix 11a

Moulds detected during micro-malting of inoculated malts in malting Run II by direct plating, percentage contamination; and MFI's.

TFm5$_2$ (F. nivale)	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination				
Field fungi					
Alternaria alternata	-	2	-	-	-
Aureobasidium pullulans	6	-	-	-	-
Fusarium spp.	-	-	-	-	-
F. nivale	26	16	-	-	2
Geotrichum candidum	12	4	26	10	-
Mucor spp.	-	-	14	30	14
Storage fungi					
Aspergillus candidus	-	-	6	-	8
A. flavus	4	2	-	-	-
A. glaucus	98	100	90	100	100
Penicillium spp.	18	10	26	14	50
MFI's					
Field fungi	0.44	0.22	0.40	0.40	0.16
Storage fungi	1.20	1.12	1.22	1.14	1.58
All fungi	1.64	1.34	1.62	1.54	1.74

- = Not detected.

Appendix 11b

Moulds detected during micro-malting of inoculated malts in malting Run II by direct plating, percentage contamination; and MFI's.

TFm7$_2$ (*G. Candidum*)	Barley after first steep	Steep out barley	Green malt	Kilned malt	Screened malt
	Percentage contamination				
Field fungi					
Geotrichum candidum	100	100	100	60	100
Mucor spp.	-	-	-	36	-
Storage fungi					
Aspergillus candidus	10	6	10	2	6
A. glaucus	86	98	80	96	100
Penicillium spp.	14	6	10	8	-
MFI's					
Field fungi	1.00	1.00	1.00	0.96	1.00
Storage fungi	1.10	1.10	1.00	1.06	1.06
All fungi	2.12	2.10	2.00	2.02	2.06

- = Not detected.

Appendix 12

Statistical analysis of results of control and inoculated malts.

Since the sample size was small, the Student t-test was used to determine the significant difference in the means of the control and inoculated malts, using the following equation:

$$t = \frac{\bar{x} - y}{s\sqrt{n}}$$

where $\bar{x}$ = mean of the test samples.

y = mean of the control samples.

s = standard deviation.

n = number of replicates.

The significance of 't' was determined from Tables (Chatfield, 1983) knowing the degree of freedom = n - 1, i.e. two, and a two tailed test.

Appendix 13

Effect of length of incubation of culture filtrate of F. nivale on glucanase activity at 37° C.

Incubation time (min.)	Absorbance (600 nm)	Activity (µg ml^{-1} glucose)	Incubation time (min.)	Absorbance (600 nm)	Activity (µg ml^{-1} glucose)
0	0	0	160	0.315	29.0
5	0.009	2.0	180	0.320	29.5
10	0.023	3.0	200	0.335	33.0
20	0.044	5.5	220	0.405	39.5
30	0.085	11.5	240	0.400	39.0
40	0.106	14.0	300	0.400	39.0
50	0.116	15.0	360	0.440	42.0
60	0.185	19.5	420	0.475	45.5
70	0.179	19.0	480	0.490	46.5
80	0.179	19.0	600	0.510	49.5
90	0.175	18.0	720	0.550	51.5
100	0.179	19.0	840	0.475	45.4
110	0.230	22.0	960	0.335	33.0
120	0.330	32.0	1080	0.330	32.0
140	0.325	30.5			

Appendix 14

Chemicals. In addition to ANALAR grade chemicals, the following compounds were used:

Compound	Supplier
Aesculin	Sigma
Arbutin	Sigma
Beta-amylase	Clodor Ltd
Chloros	A. & J. Beveridge
Elastin	Sigma
Folin phenol reagent	BDH
Hog pancreatic -amylase	Sigma
O-nitrophenol-β-D-galactopyranoside (ONPG)	Sigma